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# THE ISOLATION OF INFLUENZA A AND B VIRUSES BY CHICK-EMBRYO INOCULATIONS

by W. I. B. BEVERIDGE, F. M. BURNET[1] AND S. E. WILLIAMS[2]

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*(Accepted for publication 19th November, 1943.)*

Some irregularly positive results were obtained by Francis and Magill (1937) in isolating influenza A strains on the chorioallantois of the chick embryo, but the method was never used on any practical scale. In 1940 Burnet described the method of amniotic inoculation and showed that influenza A strains of all degrees of adaptation could be grown in this situation. Two strains were isolated by the method from natural human infections later in the same year (Burnet and Foley, 1941). In 1942 amniotic inoculation was used as the standard method in the study of a typical influenza A epidemic. Eight strains were isolated from ten cases studied early in the course of the disease. Two features were noted at the time which called for further study: (1) The amniotic fluid from infected embryos agglutinated human or guinea-pig cells but only to a very slight extent fowl cells. (2) Almost invariably only a proportion of the embryos inoculated with the same filtrate became infected; over the whole series about 50 p.c. of eggs which received material known to contain virus gave positive results. There was therefore a strong suggestion that embryos varied rather sharply in their susceptibility to infection by small doses of unmodified strains (Burnet, Beveridge, Bull and Clark, 1942).

A study of the changes in the properties of influenza A virus as it becomes adapted to growth in various regions of the embryo has been recently published (Burnet and Bull, 1943). The unadapted ("O") form, as found in amniotic fluid from a primarily infected embryo, was characterized as (a) showing a low F/G ratio (i.e., the ratio of the haemagglutinating titres observed with fowl to those with guinea-pig red cells) and (b) being unable to infect the embryo after inoculation into the allantoic cavity. With adaptation "D" phase variants appeared with F/G ratios near unity and capable of free growth after inoculation into the allantoic cavity. There were a few other minor points of difference between the two phases. In the course of work now reported it was noted that O phase strains (as well as D strains) would multiply after yolk-sac inoculation in 6-day embryos causing death in five to seven days.

During the winter of 1943 there was no epidemic of influenza in Victoria, but amongst the seasonal respiratory infections in the camps we found a proportion of both A and B influenza. It has been the usual experience that it is more difficult to isolate virus from low grade influenza prevalences of this type than from a typical epidemic (see Andrewes, 1942), so that these cases provided a valuable test for the efficiency of the chick-embryo methods of isolation. In all we have isolated four A strains and one B strain, and have failed to isolate three A and ten B strains from patients shown by subsequent serological test to have been infected with the virus in question.

[1] Work carried out by the authors with the aid of grants for research on virus diseases from the National Health and Medical Research Council and from Mr. E. Alec Cato.

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## TECHNIQUE.

Patients were instructed to gargle 10 ml. of normal saline for half a minute. Five ml. of 20 p.c. horse serum broth were added to the garglings which were then transferred to a screw capped bottle and placed in an ice-packed thermos flask for transit to the laboratory. A sample of blood was taken at the same time for serological study and a second bleeding made 12 days later.

The throat washings were filtered through sterile filter paper and the paper filtrates pooled if necessary and then passed through an Elford-type gradocol membrane filter of A.P.D. 700–800 mμ.

The technique for amniotic inoculation was essentially that described previously (Burnet, 1940) but as a rule 11-day embryos were used instead of 12-day. A minor change was the use for inoculation of capillary pipettes drawn to a fairly fine conical point and then bevelled on a fine carborundum stone. A few eggs were inoculated satisfactorily by Taylor's (1942) method but we found it impossible to obtain suitable glass covers to fit over the airspace end of the egg and none of the makeshifts tried were satisfactory. As a rule about 0·25 ml. of filtrate (not accurately measured) was inoculated into each egg. The embryos were usually examined after four days incubation at 35° C., attention being mainly directed to the amount and appearance of the amniotic fluid and its haemagglutinating power against fowl and guinea-pig or human cells. When subinoculations were made from apparently negative embryos the usual procedure was to test for sterility overnight and then pool all fluids from the one set of primary inoculations. Four embryos were inoculated amniotically each with about 0·1 ml. of the pooled fluids.

In some of the unsuccessful attempts at isolating influenza B virus 8-day embryos were inoculated amniotically. Essentially the same technique was used as for older embryos except that a finer pipette was employed and only about 0·05 ml. of fluid inoculated. With 8-day embryos there is often a considerable nonspecific mortality, up to 50 p.c. sometimes dying by the third day, so that usually eight eggs were inoculated with each filtrate. Embryos dying up to the third day were discarded, the others were examined at death or on the sixth or seventh day. Second passage from the fluids obtained was made in 11-day embryos.

Yolk-sac inoculations were made in 6-day embryos using either 0·5 or 1·0 ml. of filtrate. The eggs were candled daily and opened at death. When they had survived eight days, subinoculation was made or the series discarded. The presence of virus was detected by haemagglutination tests with an emulsion of yolk-sac. Such emulsions were normally prepared by removing about two-thirds of the yolk-sac to a sterile petri dish where it was washed with saline. After a small portion had been removed for smear, the remainder was drained and placed in a small test tube of stout glass. The membrane was ground in the tube with quartz powder using a sterile glass rod. About 1 ml. of saline was added and after complete suspension the mixture was allowed to settle for an hour or lightly spun. The densely opaque intermediate layer was found to provide suitable starting material for the serial dilutions in saline used in determining F/G ratios; the character of the agglutination was slightly modified in the higher concentrations but with a little experience no difficulty was found in reading the results.

Smears were made largely because on several occasions a filterable vibrio which did not grow on blood agar has been obtained in yolk-sacs from eggs inoculated with throat filtrates. They also provide a quick means of eliminating gross bacterial contamination as a cause of death. Castaneda's stain was found satisfactory for these purposes.

*Isolation of an A and a B strain of Influenza Virus from a Camp Outbreak.*

A relatively small prevalence of influenza-like illness occurred during June in a military camp in northern Victoria; from each of nine patients were obtained a throat washing and two specimens of blood taken with a fortnight's interval. Subsequently serological tests showed that two patients, FOL and BON, had influenza A and B respectively, whereas the other seven patients showed no rise in antibodies to influenza.

The patients showed generally uniform symptoms of influenzal type without much coryzal element but with sore throats in many cases. The two patients providing evidence of influenza virus infection can be taken as typical of the group. FOL: "Admitted three days ago with temperature 102° F., complaining of shivers, headache, and sore throat". He stated that the onset of symptoms was five days before the throat washing was obtained. BON: "Headache, shivers, fever one and a half days ago, admitted yesterday with temperature 100·5° F."

The nine throat washings were filtered through paper and four pools subjected to gradocol membrane filtration. Two pools were completely negative. The pools in which FOL was included gave 2 out of 5 positive fluids in amniotically inoculated eggs and was thereupon retested, diluted 1: 10 and 1: 100 amniotically and undiluted and 1: 10 in yolk-sacs. The results obtained are as tabulated. (Table 1).

Strains from both amniotic and yolk-sac infections were typical influenza A viruses giving F/G ratios of characteristic "O" type 10/80, 10/160 in amniotic fluids and $< 5/240$ for the yolk-sac emulsion. The serological character was checked by both haemagglutination and allantoic neutralization tests against standard ferret sera. The strain appears to be close to "Melbourne" and BEL (isolated 1942) but no finer serological analysis has yet been attempted.

TABLE 1.

*Titration of F.L.M. pool (including FOL).*

| Amniotic inoculation. | | Yolk-sac inoculation. | | |
|---|---|---|---|---|
| Undiluted | + + —† — — | Undiluted | 1·0 ml. | + — — — |
| 1: 10 | + + — | „ | 0·25 ml. | —* —* —* —* |
| 1: 100 | — — — — | 1: 10 | 0·5 ml. | — — — — |

* Subinoculation of pooled yolk-sac emulsions to amnion negative.
† Subinoculation to amnion negative.
+ (amniotic) typical haemagglutination of guinea-pig cells to a dilution of 1: 20 or more.
+ (yolk-sac) death with specific agglutination by yolk-sac emulsion.

The filtrate BON was tested alone. Eggs inoculated amniotically and opened after four and five days incubation gave negative results when tested with fowl and guinea-pig cells. Amniotic fluids from these eggs were pooled and subinoculations made by the amniotic route into further embryos. Fluids from these showed typical haemagglutination of both fowl and guinea-pig cells. In view of this positive result the first passage pool was titrated amniotically. Infections were produced in most embryos inoculated with 1: 10 and 1: 100 fluid and there were two late infections with 1: $10^3$ and one with 1: $10^4$ fluid. We have no definite knowledge as to how many of the embryos inoculated with the original filtrate became infected but small amounts of two of the first passage fluids had been kept separately. Neither of these was infective when subinoculated separately so that not more than two of four embryos can have been infected with the human material.

The positive amniotic fluids obtained from this titration reacted to approximately equal titres with fowl and guinea-pig cells. None showed a low F/G ratio such as is characteristic of primary isolation of influenza A virus. Transfer from these fluids could be made by amniotic, allantoic or yolk-sac inoculation with the development of typical influenza-virus type infection. Ferrets showed no temperature rise above 103° F. when inoculated with high titre egg fluids but a specific antibody rise was produced. Serological study by human (homologous), ferret and fowl immune sera showed that the strain was of B type showing no cross reaction with any A antisera (including "W.S.", "Melbourne", GAT, "Swine" and BEL) and significant cross reaction with Francis's type B strain "Lee". Full details of the cultural and serological characters of the strain BON will be given in a separate paper. Here it is only necessary to indicate the cross-reactions between BON and "Lee" as shown with ferret antisera (Table 2).

TABLE 2.

*Serological relationship between "Lee" and strain BON.*

| | Ferret 183 "Lee". | | Ferret 188 BON. | |
|---|---|---|---|---|
| Virus. | Normal. | Immune. | Normal. | Immune. |
| "Lee" | 300 | 1,500 | 35 | 140 |
| BON | 8 | 35 | 10 | 480 |

Titres obtained when serum dilutions tested against five agglutinating doses of virus by a modified Hirst technique.

This Table and other serological evidence to be presented elsewhere indicates clearly that while BON is related to "Lee" and must be called a B type strain, it is far from being serologically identical with the type strain. The difference is more than sufficient to eliminate any possibility that the virus BON is derived from the living attenuated "Lee" used earlier in the winter for immunization.

*Other Primary Isolations.*

It is only necessary to give a brief statement in regard to the other isolations. Strain BOR (type A) was from a pooled filtrate. There was no evidence of infection in eggs inoculated with filtrate amniotically or in the yolk-sac but subinoculations from pooled amniotic fluid and from pooled yolk-sacs were both positive, the second inoculation in each case being done amniotically. This strain therefore resembled BON in failing to produce evident primary infections.

Strain CKS, also from a pool, gave three frank infections by yolk-sac inoculation of the primary filtrate, all of O type. The amniotic inoculations done in parallel were all contaminated owing to a technical mishap. Subinoculations from the positive yolk-sacs gave quite typical findings and the strain was identified as of A type by allantoic neutralization tests.

In both instances men showing subsequent antibody rises against A virus were included in the

groups from which these pooled throat filtrates were obtained, one in BOR group, two in the C.K.S. group.

Strain HOL was obtained from a soldier with clinically typical influenza without other cases being observed in his unit. The amniotic inoculations opened at three days were apparently negative and subinoculation from one to other embryos by the same route also gave a negative result. Yolk-sac inoculations gave specific deaths on 5, 6 and 7 days and subinoculations from these eggs gave typical infections by all three routes. It was at first thought on the basis of these results that this strain was capable of primary isolation only in the yolk-sac. When this conclusion became more and more at variance with developing experience another series of eggs was inoculated amniotically with the primary filtrate which had stood for over a month at $\pm 4°$ C. Three of the five surviving embryos showed typical type O infections when they were examined at four and six days. Eggs inoculated with filtrate diluted 1: 10 gave no infections either by amniotic or yolk-sac inoculation. Since the titre of the primary filtrate must have fallen considerably since it was prepared, the failure to isolate virus in the first set of amniotic inoculations is difficult to explain. The most likely reason is to be related to the finding that we have practically never obtained visible infections in 100 p.c. of the embryos inoculated with primary human filtrates. It seems likely that only about 50 p.c. of embryos are capable of being infected by the "human" influenza A virus strains which have been current here in 1942 and 1943. Unfortunately the sporadic nature of influenza virus infections this winter has made it impossible to carry out any more detailed study of the titre of primary filtrates.

*Difficulty of Isolating Influenza B Virus.*

In a separate paper (Beveridge and Williams, 1944) an account is given of the clinical and epidemiological features of respiratory infections in Victorian camps during 1943. In order to obtain a representative sample of the infections weekly visits were paid to the most convenient camp hospital and irregular visits (depending on the incidence of infections) to more distant ones. Throat washings and blood samples were obtained from any patients who were still febrile. Many of these infections were not typical of influenza and many were only seen two, three or four days after the onset of symptoms. Titrations of first and second blood samples from these patients showed that there had been eleven cases of influenza B distributed fairly evenly throughout the winter and—except for the prevalence described in the previous section—unassociated with any epidemic.

In only one attempt (BON) did we succeed in isolating influenza B virus from these patients with serological evidence of infection. These failures may have been largely due to unsuitable material since 7 of the 11 throat washings were obtained two or more days after the onset; the only successful isolation was from one of four washings obtained 24–36 hours after onset. In an attempt to improve the results younger embryos were also used for some of the later filtrates since it had been shown that 8-day embryos were approximately 100 times as susceptible as 12-day embryos in detecting minimal amounts of nearly unadapted influenza B virus. The period of incubation before opening the eggs was also increased to six days, after we had found that very small inocula of the strain BON sometimes required this period to produce sufficient virus to be recognized by haemagglutination.

Our failures even with these refinements may have been due to the presence of strains less readily cultivable in the chick embryo than BON, or to the difficulty of obtaining virus in adequate concentration from the patient. There is no basis available for discussing the first alternative. Our experience with experimental human infections with low grade influenza virus is that only minimal amounts of virus can be obtained at the optimal period and it is possible that with the short-lasting mild infections due to influenza B in 1943 only throat washings taken at an optimal period (i.e. very soon after the onset) would contain sufficient virus to be detectable by our methods.

No attempt has been made to compare ferret and chick-embryo methods for the isolation of influenza B virus, nor has any attempt been made to combine the advantages of the two methods by inoculating ferrets with unfiltered material and transferring the infected turbinates to chick embryos after an appropriate interval. The great virtue of the amniotic method is that the virus is available for experimentation in a form which is presumably very little altered from the naturally occurring virus. If the first passage material is stored on $CO_2$ ice, large amounts of second passage amniotic fluid of high titre can be prepared from it at any time. By the ferret-mouse method not only are months of work necessary before the virus is sufficiently adapted to study in detail but the large series of passages in a new host must modify the virus considerably.

In addition to the cases which were subsequently shown to be influenza A or B over 50 other cases of the same general clinical type were similarly tested. No evidence was obtained of the growth of any virus in the embryos. Many of the throat washings were also cultured for pleuropneumonia-like organisms but none were found.

*Changes Associated with Yolk-sac Transfer.*

As far as we are aware there have been no previous reports either on the growth of influenza viruses in the yolk-sac or on the use of the method for primary isolation of the virus. We have

therefore made a number of observations on the comparative sensitivity of yolk-sac and amniotic methods and of the changes which take place in the course of yolk-sac passage of unmodified (phase O) influenza virus A.

Strain HOL may be taken as representative. This was isolated by inoculation of the primary throat filtrate into the yolk-sac of four 6-day embryos. Embryo 77748 died after seven days' incubation and gave a yolk-sac emulsion with F/G ratio $< 5/160$.

This emulsion after five days in the refrigerator was titrated wtih the results shown in Table 3.

TABLE 3.

*Comparative titration of yolk-sac HOL (O).*

| Method of titration. | Undiluted. | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ |
|---|---|---|---|---|
| Amniotic 0·05 ml. | | | 30/240 20/160 | 5/40 |
| Yolk-sac 0·5 ml. | | | 12/30 50/240 0/25 | 0/50 0/5 + + |
| Allantoic 0·05 ml. | 10/15 20/35 — — | + — — — | — — — — | — — — — |

| Method of titration. | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
|---|---|---|---|
| Amniotic 0·05 ml. | 20/160 15/120 25/160 + | 15/80 15/100 + — | 30/240 — — — |
| Yolk-sac 0·5 ml. | 0/200 0/320 — | 0/10 — — — | |
| Allantoic 0·25 ml. | — — — — | | |

The ratios 30/240 etc. each represent a positive fluid or emulsion the F/G ration of which is shown.
+ positive haemagglutination, F/G ratio not done.
— fluid shown no agglutination of guinea-pig cells.

We can summarize the results as indicating first that the amniotic method is the most sensitive indicator of virus. Allowing for the fact that a ten times greater inoculum is used for yolk-sac eggs the amniotic method is approximately 100 times as sensitive at detecting O type virus as the yolk-sac. Adopting the criteria used in former work on the O–D transformation the emulsion 77748 contains only about one D phase virus particle to $10^5$ or $10^6$ O particles since infection occurs only in allantoic eggs inoculated with 1: 10 or undiluted.

If the F/G ratios from positive yolk-sac eggs are examined it will be noticed that the higher dilutions give values in which the fowl cell titre is rated O. These emulsions even when diluted only 1: 2 give a completely negative reading whereas amniotic fluid O virus of comparable titre when tested with guinea-pig cells always shows a certain amount of agglutination with fowl cells. The difference appears to be largely due to a physical effect of the yolk constituents in the emulsion since a positive amniotic fluid mixed with inactive yolk-sac emulsion shows greatly weakened fowl-cell agglutination. The values 12/30 and 50/240 shown with yolk-sacs from the 1: 100 dilution must similarly be taken as equivalent to considerably higher D values.

In order to establish whether the O–D relationship was shown in the same fashion with yolk-sac passage as with amniotic passage, the yolk-sac passage of HOL was continued for five generations. The essential findings are shown in Table 4.

Features in this series are: In the first titration of 78533 (not shown in the Table) a large number of embryos died without showing agglutination of either fowl or guinea-pig red cells.

TABLE 4.

*Yolk-sac passage of an A strain HOL.*

| No. of passage and source of inoculum. | Dilution of stock yolk-sac emulsion. $10^{-2}$ | $10^{-4}$ | $10^{-6}$ | $10^{-8}$ |
|---|---|---|---|---|
| 1st Y.S. 77748 | $O^4$ $O^4$ $O^5$ | $O^6$* $O^7$ | $O^8$ $O^8$ — | — — — — |
| 2nd Y.S. 78533 (ether treated) | $O^5$* $O^6$ | | | |
| 3rd Y.S. 78889 | $D^4$ $D^4$ $D^4$ | $D^4$ $D^4$ $D^4$ | $D^4$ $D^5$ $O^5$* | |
| 4th Y.S. 79199 | | $O^4$ $O^4$* $O^4$ | | $O^4$ $O^4$ — |
| 5th Y.S. 79730 | $D^3$ $D^4$ $D^4$ | $O^4$ $O^5$ $O^7$ | $D^6$ $O^5$ $O^7$ | |

$O^7$ Embryo died after 7 days incubation giving a yolk-sac emulsion with F/G haemagglutination ratio 0/320 or similar value.
$D^4$ Embryo dead at 4 days giving yolk-sac emulsion with F/G 40/120 or similar value with definite fowl-cell agglutination.
* Yolk-sac emulsion used for next passage.

These deaths were possibly due to the presence of a filterable vibrio which we have isolated six times from about twenty throat filtrates tested by yolk-sac inoculation. The emulsion 78533 was therefore diluted 1: 100 in a mixture of saline and nutrient broth, shaken with excess ether and left for three hours in the refrigerator in a stoppered vessel. After most of the ether had been removed from the aqueous layer by evaporation at room temperature, yolk-sac inoculations were made and from embryo 78889 dead at five days an emulsion giving F/G 5/140 was obtained and smear showed no vibrios. This yolk-sac emulsion apparently contained a large proportion of D virus since early deaths occurred right through the series and only one embryo inoculated with $10^{-6}$ dilution gave O type virus. Subinoculation of this O fluid (79199) at $10^{-4}$ and $10^{-6}$ gave the expected uniformly O type yolk-sac emulsions and typical findings were again obtained in the fifth passage. In all essential respects therefore passage of O phase virus by yolk-sac inoculation reproduces the findings obtained by amniotic passage in our earlier work.

## DISCUSSION.

The results obtained from this study of the primary isolation of influenza viruses by chick-embryo inoculation has fully confirmed the findings obtained in 1942 in regard to influenza A. All A strains isolated have been in the O phase and have regularly changed to the D phase when subinoculated in normal fashion. A fresh development has been the demonstration that yolk-sac inoculation can be used for primary isolation although it is decidedly less sensitive than the method of amniotic inoculation. At least a proportion of embryos inoculated amniotically will respond to one-hundredth of the amount of virus necessary to induce signs of infection in the yolk sac. The behaviour of O phase virus in the yolk sac is in all essential respects similar to that in the amniotic cavity. Mutation to D phase occurs, but by passage at high dilutions the strain can be maintained more or less indefinitely in the O phase.

We have had one success in isolating influenza B virus by the amniotic method, but in view of the considerable number of failures we feel unjustified in doing any more than record the isolation here. A full account of the properties of this B strain is given in another paper (Burnet, Beveridge and Bull, 1944).

With the development of chick embryo methods for primary isolation of influenza virus an opportunity is now available for detailed study of the amount and character of virus present in human throat washings at different stages of infection. We had hoped to make such a study during 1943, but the fact that only a very small proportion of the respiratory infections studied were due to influenza A or B viruses and that we had only very limited storage facilities at —76° C. made it a practical impossibility to do so.

The most important practical requirement is to determine whether 11-day embryos inoculated amniotically are uniformly susceptible to influenza virus A as it occurs in human throats. On this point we have unsystematic but rather convincing evidence that there are differences in susceptibility. It has been very rare to find all the embryos inoculated with one filtrate giving positive results. In tests with 14 filtrates each of which gave one or more positive results only 30 of the 56 embryos which survived four days or more gave virus as indicated by agglutination of guinea-pig cells by the amniotic fluid. The results therefore suggest that about 50 p.c. of embryos are intrinsically more susceptible than the rest. If this is so we must face the possibility that under certain circumstances the proportion of insusceptible embryos might be much higher and render the method ineffective.

Only when the sensitivity of the method has been accurately determined will it be possible to approach the questions of the uniformity of the virus population found in the throat washings of an influenza patient and the amount of virus present at different periods of the disease. As has been stressed in an earlier paper (Burnet and Bull, 1943), practically all experimental work on influenza has been carried out with virus whose character is far removed from that of the actual human-pathogenic virus. A full understanding of the human disease can

hardly be expected until the technique of dealing with the virus in its unmodified form has been fully developed and exploited.

## SUMMARY.

During 1943 four strains of influenza virus A and one of influenza virus B were isolated by chick-embryo inoculation from sporadic cases of respiratory infection in Victorian military camps.

Yolk-sac inoculation of human material gave three primary isolations of influenza virus A but comparative experiments indicated that amniotic inoculation was a considerably more sensitive method.

All A strains were isolated in the O phase. On passage either by amniotic or yolk-sac methods mutation to the D phase occurred. Pure O phase virus could be maintained by passage at limiting dilutions.

Only one successful isolation of influenza B virus was made out of eleven throat washings from patients subsequently shown to have been infected with the virus. Possible reasons for these failures are discussed.

The potentialities of the embryo inoculation methods for the study of influenza virus in its natural (phase O) state are discussed.

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# STUDY OF A STRAIN OF INFLUENZA B VIRUS ISOLATED BY CHICK-EMBRYO INOCULATION

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Influenza virus B has now been shown responsible for at least two large epidemics as well as smaller prevalences of respiratory infection, but only a few strains of the virus have been isolated. The strain (Lee) isolated by Francis in 1940 has been used by most workers as the standard type of the virus. Records of other isolations are as follows: Magill (two strains, New York) 1940, Eaton and Beck (strain "Montgomery," California) 1941, Nigg *et al.* 1942 (two strains isolated from material stored since May 1939, from a mental hospital in New York State), Gordon, 1942 (strain 572 from Cuban epidemic, 1940) and Taylor *et al.* 1942 (Argentina, 1941).

All these strains with the exception of Taylor's were isolated by repeated ferret and mouse passage, usually with very little to be seen in the early passages. Taylor's strain was initiated in hamsters. All strains have been finally established in mice in a state far removed from the original human virus. With the isolation of a strain of influenza B virus by amniotic inoculation an opportunity became available to study the character of the virus in a state very close to the original. We were particularly interested in an attempt to characterize the early stages of adaptation to laboratory maintenance in chick embryos or mice in order to compare the changes in a B strain with the well marked O-D transformation which we have recently described for A strains (Burnet and Bull, 1943). In the course of this work we have examined the effects of inoculating chick embryos by amniotic, allantoic and yolk-sac methods and have studied the process of adaptation to mouse-lung propagation. The serological relationship to "Lee" has been studied, using immune fowl and ferret antisera with tests by haemagglutination and by allantoic inoculation methods.

### *Adaptation of Strain BON to Growth in Chick Embryo Tissues.*

In the accompanying paper (Beveridge, Burnet and Williams, 1944) the circumstances associated with the isolation of influenza B virus from patient BON in a Victorian military camp are described. Here we need only recapitulate briefly that the primary filtrate from the throat washings failed to produce any evidence of infection in 11-day embryos inoculated amniotically and opened after four and five days incubation at 35° C. Pooled amniotic fluid from these embryos (78205–9) was found to induce infection on subinoculation by the amniotic route and a full titration of the pool was then carried out in 11-day embryos. The results are shown in Table 1. The features of this Table are first the evidence of delay in attaining a detectable haemagglutinating titre especially in the higher dilutions and secondly the number of failures to produce visible infection in several eggs inoculated with relatively low dilutions. Eggs inoculated with the same material (78205–9) diluted 1: 10 by either allantoic or yolk-sac methods gave no evidence of infection when opened after four and nine days incubation respectively.

As a starting point for further investigation fluid 78056, i.e. the only visibly positive fluid obtained with $10^{-4}$ dilution, was used. By analogy with an A strain this should correspond to an O-phase fluid but the results of differential haemagglutination tests gave an F/G ratio of 25/20. Table 2 gives the results of an incomplete titration of this fluid 78056 by amniotic, allantoic and yolk-sac inoculation in chick embryos.

[1] Work carried out by the authors with the aid of grants for research on virus diseases from the National Health and Medical Research Council and from Mr. E. Alec Cato.

TABLE 1.

*Titration by amniotic route of apparently inactive first passage fluid strain BON.*

| Dilutions. | Day examined. | Result. |
|---|---|---|
| $10^{-1}$ | 3 | + + + + + — — |
| $10^{-2}$ | 3 | + + + + — — — |
| $10^{-3}$ | 3 | — — — |
| | 5 | + + — — |
| $10^{-4}$ | 3 | — — — |
| | 6 | +* — — — |

+ agglutination of fowl red cells by amniotic fluid tested at time shown.
— no agglutination.
* embryo 79056 used for subsequent passage, the F/G ratio was 25/20.

TABLE 2.

*Partial titration of second passage amniotic fluid 78056 by various routes.*

| Dilution. | Amniotic. | Allantoic. | Yolk-sac. |
|---|---|---|---|
| $10^{-2}$ | 3 day + + + (1,200/500) | 3 day — — — | 7, 8, 8, — |
| | | 4 day + — — | 7, 8, — |
| $10^{-4}$ | 3 day + + — | | |
| $10^{-6}$ | 3 day — 4 day + + (350/120) | | |
| $10^{-8}$ | 3 day — 4 day + (40/10) | | |

+ Specific haemagglutination, representative F/G titres shown in parenthesis.
— No evidence of infection.
Under yolk-sac, figures indicate days to death with positive haemagglutination in yolk-sac emulsion.

In summary this fluid was active by amniotic titration in 11-day embryos to a titre of at least $10^6$ but required four days to reach detectable titre with the higher dilutions. When diluted only 1: 100 it failed to infect allantoically at three days, one embryo of three becoming positive on the fourth day and in the yolk-sac death occurred late after seven or eight days incubation with some failures to infect.

With passage by either amniotic or allantoic routes the strain soon developed greater activity as shown by (1) a higher allantoic titre which however has not reached the same level as is shown in amniotic titrations, (2) a more rapid lethal effect after yolk-sac inoculation, and (3) increased power to produce pulmonary consolidation in mice inoculated intranasally.

TABLE 3.

*Comparative titration of an adapted BON allantoic fluid (82781–3).*

| Dilution. | Amniotic. | Allantoic. | Yolk-sac. | Mice. |
|---|---|---|---|---|
| $10^{-1}$ | | | | $4^7$ $4^8$ $4^8$ 3 |
| $10^{-2}$ | | | †3 †3 †4 †5 | 3 3 3 |
| $10^{-3}$ | | | | 1 1 1 0 |
| $10^{-4}$ | | | †7 †7 †7 †7 | |
| $10^{-5}$ | + + + + | + + — — | | |
| $10^{-7}$ | + + + + + — | — — — — | | |
| $10^{-8}$ | + + + + — — | — — — — | | |

Amniotic, allantoic and yolk-sac titrations: symbols as Table 2.
Mice lesions shown by usual convention, $4^7$ indicating death at 7 days with complete lung consolidation, 3, 2, 1 diminishing degrees of consolidation, 0 no lesions.

As an example of a well-adapted fluid, the comparative titration of a pooled allantoic-fluid virus which had been passed successively through five amniotic, one mouse and five allantoic passages may be tabulated (Table 3). This fluid 82781–3 had haemagglutination titres of 320 with fowl cells, 25 with guinea-pig cells.

Changes in the haemagglutination F/G ratio were not sufficiently definite to allow any definition of O and D forms analogous to those obtained with influenza A. Table 2 for instance

shows that 2nd passage amniotic fluids all show a high F/G ratio while later passage allantoic fluids may show ratios higher still, e.g. 320/25 for the fluid used in the titrations of Table 3. In none of the amniotic titrations have we found one type of F/G ratio shown with fluids from egg inoculated with higher dilutions and another with those from embryos inoculated with lower dilutions.

*Relative Susceptibility of Embryos of Different Ages to Virus Inoculated Amniotically.*

In seeking a more sensitive method of isolating influenza and possibly other viruses we have several times tried inoculating amniotically embryos younger than the standard 11 or 12 days. Experiments with O phase influenza virus A showed that 12-day embryos were slightly more sensitive than 8-day embryos in detecting minimal amounts of virus.

Similar experiments with BON using first or second passage amniotic fluids as inoculum have shown on the other hand that the younger eggs are the more susceptible. First passage material (78205–9) stored for some weeks, and diluted 1: 20 was used to inoculate 10, 11 and 15-day embryos amniotically. Samples of amniotic fluid were tested for haemagglutinins after three and four days incubation. At three days the proportions of positive findings were: 10-day embryos 3/4 +, 11-day 0/5 +, 15-day 0/5 +; at four days 10-day 4/4 +, 11-day 3/5 +.

TABLE 4.

*Titration of second passage BON 81529 amniotically in embryos of different ages.*

| Age of embryo when inoculated. | Dilutions of virus. $10^{-6}$ | $10^{-8}$ | $10^{-7}$ |
|---|---|---|---|
| 12 days | $\frac{1}{5}$ | $\frac{0}{9}$ | $\frac{0}{4}$ |
| 8 days | $\frac{3}{4}$ | $\frac{8}{10}$ | $\frac{5}{7}$ |

The denominator in the fractions represents the number of embryos which were inoculated and survived four days; the numerator is the number which gave a positive haemagglutination test on the fourth day.

A more extensive test was made with a second amniotic-passage fluid which gave no growth when inoculated into the allantoic cavity at 1: 10 and 1: 100 dilutions and was therefore still in essentially unadapted form. The results of testing 8- and 12-day embryos with high dilutions of this fluid are shown in Table 4 the results given being those obtained after four days incubation. A few 10-day embryos were also inoculated in this series. They gave irregular results which however included two positive fluids from three surviving embryos given the highest virus dilution ($10^{-7}$). In another test 10-day embryos gave the same results as 8-day ones.

In the course of this experiment the amniotic fluids of some of the embryos were sampled at four days and the eggs then returned to the incubator to be retested at six days. In two instances (one 8-day and one 10-day embryo) fluids had changed from negative to positive in the interim.

A similar experiment with an adapted strain of BON (that used in the titrations shown in Table 3) showed that the 8-day eggs gave 8/8 positive results at $10^{-7}$, 1/5 at $10^{-8}$, 0/6 at $10^{-9}$ against the 11-day results shown in Table 3 which include 4/6 positive at $10^{-9}$.

It seems therefore that the detection of unadapted virus of BON type the use of 8- or 10-day embryos provides a method about 100 times more sensitive than the standard 12-day embryos. The fluid used had a haemagglutinin titre of 800 which in an adapted virus would correspond to an infective titre about $10^{-7}$. The 12-day embryos were therefore abnormally insensitive to this virus while the 8-day embryos were susceptible to the full extent expected. This virus had had two amniotic passages before being tested so that it had presumably undergone a certain amount of adaptation. It cannot therefore be assumed that 8-day embryos would be as highly susceptible to the virus of influenza B as found in human throats. All the results would however suggest that the 8-day embryos would manifest similar advantages over 12-day ones for primary isolations of this virus. So far no further isolations of influenza B have been made with either 8-day or older embryos.

*Yolk-sac Passage of BON.*

In previous work we have found that O phase influenza A virus will multiply in the yolk-sac and that under favourable circumstances primary isolations may be made by this method. As shown in Table 2 the second amniotic passage of BON gave fluid capable of producing 5 out of 7 fatal infections in 6-day embryos inoculated by the yolk-sac route. Emulsions were prepared

from these yolk-sacs by gentle washing in saline followed by grinding with quartz powder and adding 2 ml. of saline to each yolk-sac. Such emulsions after light centrifugation gave an opaque supernatant fluid with a variable haemagglutinin titre, the value always being higher for guinea-pig than for fowl cells. Although all our evidence suggests that yolk-sac passage allows BON to remain in the unadapted state, the consistently low F/G ratio is mainly due to a physical effect of the yolk on the agglutination and is shown though less markedly when adapted BON is grown in the yolk-sac.

TABLE 5.

*Yolk-sac passage of BON.*

| Passage. | | Time, of death, days. | F/G ratios haemagglutination. |
|---|---|---|---|
| 1 | 78056 | 7, 8, 8, 8, 8, 8 | 0/0, 20/80, 10/15, 0/0 |
| 2 | 80699 | 7, 7, 8 | 5/8, 40/80 |
| 3 | 81225 | 6, 6, 6, 6, 6, 7 | 90/320, 10/20, 80/160, 100/200 |
| 4 | 81724 | 4, 5, 5, 5 | 0/0, 0/0, 0/0, 0/0 |
| 5 | 82108 | 4, 4, 5, 5, 5, 5 | 0/0, 10/20, 8/10, 12/15 |
| 6 | 82295 | 5, 5, 6, 6, 7 | 0/5, 0/0, 18/90, 8/25 |
| 7 | 82737 | 6, 6, 6, 6 | 0/0, 0/0, 0/0, 0/0 |
| 8 | 83073 | 5, 5, 6, 6 | 0/20, 0/5, 15/40 |
| 9 | 83264 | 6, 7, 7, 8 | 40/120*, 0/0, 5/15, 8/15 |

* Yolk-sac 83528 used for titration shown in Table 7.
The F/G ratios are shown in the same order as the deaths in the preceding column.

Passage from yolk-sac to yolk-sac, using 0·5 ml. of 1: 100 dilution of yolk-sac emulsion made as described, was carried on for ten passages. Throughout the series the virus remained approximately constant in character without evidence of adaptation to chick tissues. At most passages more or less complete amniotic and allantoic titrations were made, and on two occasions tests in mice. The time of death of each embryo inoculated in the yolk-sac and the F/G haemagglutinin values were determined. Table 5 shows the times of death and haemagglutinin titres obtained.

TABLE 6.

*Immunization of mice by yolk-sac passage material.*

| Initial inoculum. | | Result of test 3 weeks later. | Initial inoculum. | | Result of test 3 weeks later. |
|---|---|---|---|---|---|
| BON 81724 | $10^{-1}$ | 3, 3, 3, 2 | BON 82737 | $10^{-2}$ | 3 |
| „ | $10^{-2}$ | 3, 3, 2 | „ | $10^{-1}$ | 1, 1, 1 |
| „ | $10^{-3}$ | 1, 0 | „ | Undil. | 0, 0, 0, 0 |
| „ | $10^{-4}$ | 0, 0, 0, 0 | | Undil. | 0, 0 |
| „ | $10^{-5}$ | 0, 0, 0, 0 | | Controls | 3, 3, 3, 2 |
| | Controls | 3, 3, 3 | | | |

All the emulsions tested gave amniotic infections to a titre somewhat below what would be expected from the haemagglutinin results, with the characteristically slow development of haemagglutinating fluids at high dilutions. Allantoic results were predominantly negative but always with an occasional positive result appearing in some of the lower dilutions. The fourth and seventh passages were tested in mice. Both showed high haemagglutinin F/G ratios (90/320 and 60/120 respectively) but only the first produced any lesions in mice. These were very small and inconstant and produced no illness. When the mice were subsequently tested for immunity with "adapted" allantoic fluid of the same strain, the results in Table 6 were obtained.

TABLE 7.

*Amniotic and allantoic titrations of ninth yolk-sac passage of BON.*

| Virus dilutions. | Amniotic titration. 4 days. | Amniotic titration. 5–7 days. | Allantoic titration. 3 days. | Allantoic titration. 4 and 5 days. |
|---|---|---|---|---|
| Undil. | | | + + − − | + + − − |
| $10^{-1}$ | | | − − − − | + − − − |
| $10^{-2}$ | | | − − − − | − − − − |
| $10^{-3}$ | + + − − | | − − − − | + − − − |
| $10^{-4}$ | + + + + | | + − − − | + − − − |
| $10^{-5}$ | + − − − | | − − − − | − − − − |
| $10^{-6}$ | | + + − − | | |
| $10^{-7}$ | | + − | | |

It is clear that mice given the lower dilutions intranasally were immunized against a moderate dose of the same virus in a more virulent form.

After nine yolk-sac passages the strain still showed the characteristic features of the unadapted material, namely a slow development in the amniotic cavity requiring five to seven days to produce positive haemagglutination from minimal infecting doses and almost complete inability to grow in the allantoic cavity. The results obtained by titrating yolk-sac 83528 (9th passage) are shown in Table 7.

*Reactions in Mice and Ferrets.*

Although high titre allantoic fluid produces typical consolidation in mice inoculated intranasally we have so far failed to establish a mouse passage strain. When transfers were made at 2-day intervals the virus was lost after six passages. From the haemagglutinin titrations that we carried out on lung emulsions a maximal titre from infections with allantoic fluid is not reached till about the fifth day. Passage at this interval gave more promise but after five passages the virus was found to have been replaced by a pleuropneumonia-like organism. This has been known to be present in our stock mice for the past four years but has never given trouble when all stock influenza virus passages were made for two days only. The most active fluid we have tested, an amniotic fluid pool with haemagglutination F/G 200/40, gave five out of six deaths at $10^{-3}$ and was fatal in all lower dilutions. A titration of allantoic fluid 82781–3 in mice is shown in Table 3.

Two ferrets were inoculated with high titre fluids intranasally, neither showed significant temperature reactions. Ferret 188 inoculated with third amniotic-passage fluid, F/G 320/120, gave a maximal temperature of 103·3° F. about 24 hours after inoculation and another minor rise at about 66 hours to 103·2°. Ferret 189 ran an atypical temperature between 103° and 104° and died eleven days after inoculation from causes unrelated to the experiment. Ferret 188 gave a satisfactory immune serum but when one considers the massive dose given it becomes highly improbable that the original human material would have given any temperature or immunological response in the ferret.

*Serological Character of Strain BON.*

The strain BON is by no means serologically identical wtih the type strain "Lee" B but it is clearly nearer to this than to any A strain. Since we have no mouse-adapted substrain all our immunological tests have been made either by haemagglutination methods or by allantoic inoculation in chick embryos (Hirst, 1942; Burnet and Beveridge, 1943).

*Haemagglutination tests.* In our previous work with "Lee" we have frequently found difficulties from the high nonspecific inhibition shown by normal ferret or human sera against some batches of virus. Wherever possible we have therefore included normal (or pre-immunization) serum from the same ferret or person as well as the immune sera. With BON this nonspecific inhibition was much less frequently observed.

In tests with ferret sera immune to the A strains "Melbourne", "W.S." and Swine 15, and two representative Victorian A strains 1942 BEL and 1943 CKS, no effect whatever was shown against BON by any dilutions above 1: 20 and the results need not be tabulated. Comparison with "Lee" is shown in Table 8.

TABLE 8.

*Comparison of BON and "Lee" with immune ferret sera.*

| Ferret sera. | Titres against virus. "Lee." | BON. |
|---|---|---|
| 183 Normal | 300 | 8 |
| 183 "Lee" | 1,500 | 35 |
| 184 "Lee" | 1,800 | 30 |
| 188 Normal | 35 | 10 |
| 188 BON | 140 | 480 |

Titres represent serum dilution which mixed with 5 agglutinating doses gives weak partial (+) agglutination.

Fowl immune sera gave similar results. Fowl 22 immunized with "Lee" gave titres of 1,500 against "Lee" and 100 against BON; while Fowl 38 immunized against BON gave titres of 480 against "Lee" and 800 against homologous BON.

Human immune sera which were available for comparison included first and second bleedings from the patient providing the virus and from a number of subjects experimentally immunized with strain "Lee". We had also a number of first and second bleedings from patients showing serological evidence of infection with influenza B during 1943. The results of haemagglutination tests on these various sera against "Lee" and BON viruses are combined in Table 9.

TABLE 9.

*Comparison of human sera against "Lee" and BON.*

Patients with natural B infection, 1943. Subjects immunized with B ("Lee"), 1942.

Serum titres (first and second bleedings) against viruses shown.

| Patient. | "Lee." | BON. | Subject. | "Lee." | BON. |
|---|---|---|---|---|---|
| BON | 80–160 | 20–160 | JOH | 60–320 | 10–240 |
| WHE | 40–240 | < 10–240 | WEB | 35–120 | 60–150 |
| KEL | 20–100 | < 10–160 | MIL | 80–240 | < 10–< 10 |
| KAN | 40–100 | 15–160 | DOW | 15–300 | < 10–30 |
| STE | 60–80 | 15–60 | | | |

The 1943 patients show more definite antibody rises against BON than against "Lee" mainly because of the lower initial titres for which nonspecific inhibition of "Lee" is mainly responsible. The sera from subjects immunized intranasally with attenuated "Lee" show greater discrepancies. These sera were chosen from a larger group, all immunized with mixed A and B attenuated virus because they showed a sharp rise against "Lee" with very little or no rise against A. It will be noticed that the last two, MIL and DOW, show a rise highly specific for "Lee".

*Allantoic antibody tests.* Tests were made using the technique described by Burnet and Beveridge (1943) with first and second serum samples from the patient BON, against the strains "Lee" and BON. The results in Table 10 show that by this method there are rather sharp immunological differences between the two strains. The highly specific character of neutralization as judged by this technique has already been described for influenza A subtypes (Burnet and Beveridge, 1943).

TABLE 10.

*Allantoic titration of homologous human serum against BON and "Lee".*

| Virus and titre. | | Serum I. | Serum II. |
|---|---|---|---|
| "Lee" | 6·2 | 6·2 (0) | 4·0 (2·2) |
| BON | 6·2 | 2·7 (3·5) | — 2·0 (8·2) |

All titres expressed as logs. Under serum I and II the apparent titres of virus-serum mixtures are shown first, with the amounts of virus neutralized by undiluted serum shown in brackets. See Burnet, 1943.

Cross tests by the allantoic method with immune ferret sera showed a high degree of specificity. The full result of the experiment is shown in Table 11 to bring out a point we have often observed, viz. that with neutralization by related but not homologous antisera irregularities in the series are almost invariable; homologous reactions are clear cut.

The results of the whole serological investigation class the strain as of type B but it seems to bear much the same relationship to Francis's strain "Lee" as exists between the major subtypes of influenza A virus.

TABLE 11.

*Cross tests of ferret sera by allantoic method.*

| Serum-virus mixtures. | Ferret 184 ("Lee"). Virus "Lee". | Ferret 184 ("Lee"). Virus BON. | Ferret 188 BON. Virus "Lee". | Ferret 188 BON. Virus BON. |
|---|---|---|---|---|
| S/5 + V | + + + − | | | + + + − |
| S + V | − − − − | | + + + − | − − − − |
| S + V $10^{-1}$ | − − − − | + + − − | + + + + | − − − − |
| S + V $10^{-2}$ | | + + + + | + + + + | |
| S + V $10^{-3}$ | | − − − − | − − − | |
| S + V $10^{-4}$ | | + + − − | + + + − | |
| S + V $10^{-5}$ | | | + + − | |

## DISCUSSION.

The characteristics of the strain BON of influenza virus B during the early stages of its isolation and propagation contrast sharply with those of strains of influenza A. As no other strains of B type virus have been isolated by embryo

inoculation methods we cannot say whether the behaviour of BON is characteristic of B strains generally or not. As all A strains that we have isolated in 1942 and 1943 have shown essentially uniform behaviour it is reasonable to think that BON may be a representative sample of its group.

It will be remembered that A strains are isolated in the O form with a very low F/G haemagglutination ratio but that mutation regularly occurs to the D phase with an F/G ratio near unity and greater speed of growth in the amniotic cavity. In addition to some other minor differences D strains infect to full titre in the allantoic cavity, O strains not at all.

With the B strain no fluids giving haemagglutination ratios much below unity have been observed. The difference shown with a number of yolk-sac emulsions in favor of guinea-pig cell titres is mainly or entirely due to a physical effect of the yolk emulsion. With allantoic passage of BON one tends to obtain fluids with a very high F/G ratio, e.g., 320/25, but it has not been possible to demarcate distinct phases on a basis of F/G ratios.

The differences between unadapted strains and strains which have become adapted to allantoic passage are considerable, but more quantitative than qualitative. They may be described for each type of titration as follows.

*Amniotic*: With high dilutions it may take six days before unadapted virus reaches sufficient concentration in the amniotic fluid to give positive haemagglutination. There is less delay with adapted virus. With early passage material 8- or 10-day embryos give higher titres than 12-day embryos; no such advantage is shown with adapted virus.

*Allantoic*: Unadapted virus fails to grow in the allantoic cavity, but in almost every titration there are occasional exceptions more frequent with lower than with higher dilutions. Adapted virus multiplies freely but does not reach the titre shown in the amniotic cavity and "delayed positives" appearing after three days are fairly common.

*Mouse intranasal*: Unadapted (yolk sac) virus produces no pulmonary lesions but immunizes mice against virulent material. Adapted virus (allantoic fluid) produces typical pulmonary consolidation, with macroscopic lesions up to a dilution of $10^{-3}$. It has not yet been possible to adapt the strain to indefinite mouse-to-mouse passage.

*Yolk sac*: Unadapted virus kills 6-day embryos in five to eight days at moderate (1:100) dilution. Practically no change in character is evident with ten passages by this route, presumably indicating that the original material grows as well as any variant in this situation. Adapted virus at 1:100 dilution kills in three to five days.

The impression one receives throughout in working with the strain BON is its relative slowness in initiating infection and in liberating virus into the cavity or tissue concerned. This may be perhaps correlated with the non-epidemic character of influenza B at the present time in Victoria.

Serologically our strain BON is not very close to the only other B strain ("Lee") that we have available for comparison. The difference between the two is of the same order as that separating the A strains "Melbourne," "W.S." and GAT. As with these, ferret and fowl immune sera are more specific than human, but a proportion of human sera also shows sharp differences between the subtypes. The relative uniformity of the serological tests with patients infected with influenza B during the present year makes it likely that the strains concerned have been of the same serological character as BON.

SUMMARY.

A strain BON of influenza B virus has been isolated by amniotic inoculation in chick embryos and the changes associated with adaptation to growth in chick tissues have been studied.

Multiplication of the virus takes place more slowly than with influenza A and no discontinuous change from unadapted to adapted state has been clearly demonstrated.

In the unadapted state the virus fails to multiply after inoculation into the allantoic cavity but this capacity can be developed by passage.

Eight- and ten-day embryos are much more sensitive than are 12-day ones in detecting limiting dilutions of unadapted BON virus.

Yolk-sac passage can be carried for at least nine generations without demonstrable change in the unadapted character of the virus.

Serologically the strain is only distantly related to the standard B strain "Lee."

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# THE INFLUENCE OF TEMPERATURE ON THE ELIMINATION OF DIAPAUSE FROM THE EGGS OF THE RACE OF *AUSTROICETES CRUCIATA* SAUSS. (ACRIDIDAE) OCCURRING IN WESTERN AUSTRALIA

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*(Accepted for publication 21st December, 1943.)*

Plagues of the grasshopper *Austroicetes cruciata* Sauss. have been recorded from Western Australia (Jenkins, 1937), from South Australia (Andrewartha, 1939) and from New South Wales (Key, 1938). Plagues are restricted to well-defined areas which occur on the borders of the wheat belts in these States. The climates of the infestation areas in South Australia and New South Wales are broadly similar with respect to the temperatures prevailing during the winter and spring. But the climate of the infestation area in Western Australia differs from that in the other two areas in that the minimum temperatures prevailing during May, June and July are higher than those experienced in the infestation areas in the eastern States. These are the important months for the elimination of diapause from the eggs of *A. cruciata* (Birch, 1942).

A consideration of these differences in the climates of the two areas led to the postulate that the elimination of diapause from the eggs of the grasshopper occurring in Western Australia should proceed at temperatures higher than those required to eliminate diapause from the eggs of the eastern race.[1] To test this hypothesis, eggs of *A. cruciata* were collected from Merredin, Western Australia, and used for the experiments, reported below, in which the eggs were exposed to the same series of "low" temperatures as had previously been used in experiments with eggs of the eastern race (Andrewartha, 1943). "Low" in this connection refers to temperatures which are below the range at which the embryo grows normally (Birch, 1942).

## EXPERIMENTAL METHODS AND RESULTS.

The eggs were collected in the field at Merredin in September, 1942. They were stored at room temperature until December 4. They were then separated from the pods and placed in incubation cages made from celluloid with floors of plaster of paris (as described by Birch, 1942). The surface of the plaster was kept moist by standing the cages in petri dishes containing a little water.

Control batches of eggs were kept in the incubator continuously for six months at 25° C. and 30° C. The remainder of the eggs were exposed to 25° C. for 18 hours and to 13·3° C for 6 hours each day: this treatment promoted the growth of the embryo. With half the eggs this treatment was continued for 53 days, with the other half for 63 days. During this time the embryo developed to about the stage shown in Fig. 10 of Steele (1941): that is, the eyespots were distinctly

[1] The grasshopper *A. cruciata* occurring in Western Australia is considered to be a distinct race from *A. cruciata* in the eastern States. Although no work has been done to determine whether the differences in the eggs collected from the different regions are inherited, there is no reason to expect that they are not. The area where *A. cruciata* is found in Western Australia is quite isolated from the area where this species occurs in eastern Australia. Dr. K. H. L. Key has informed me in a letter that there are certain differences in external morphology which can be detected in series of specimens from the two regions.

visible in the posterior fifth of the egg and the embryo was about half as long as the egg.[2] For convenience in description, the eggs which had been exposed to the alternating temperatures for 53 days will be referred to below as having reached stage "A", and those exposed for 63 days as having reached stage "B".

Each of these groups of eggs was further subdivided into ten batches which received the following treatments. One batch was incubated at 25° C. for a control. Three batches were placed at 6·5° C., three at 10·0° C., and three at 13·3° C. One batch was left at each temperature for 30 days, another for 45 days, and the third for 60 days. At the conclusion of the period at these constant low temperatures the eggs were incubated at 25° C. Each treatment was replicated five times; 1,750 eggs were used for the experiment. The extent to which diapause had been eliminated by the various treatments was measured by the number of nymphs which hatched while the eggs were being incubated at 25° C.

One nymph (equivalent to 1·3 p.c. of the eggs) emerged from the control batch of eggs which was incubated at 25° C. after being exposed to alternating temperatures for 63 days. No nymphs emerged from any of the other controls, although from 70–90 p.c. of the eggs in the various controls were apparently still alive at the end of the experiment. Very little development occurred in the eggs which were incubated at 25° C. and 30° C. continuously throughout the experiment (i.e. for six months). None of the embryos developed to the stage at which eyespots become visible.

The numbers of nymphs (expressed as a percentage) which emerged from all the treatments, other than controls, are summarized in Table 1. For the purposes of statistical treatment it was necessary to transform these data to the inverse sine, i.e. $\sin^{-1}\sqrt{P/100}$ (Cochran, 1938). Consequently the figures given in Table 1 are not quite the same as would be given by calculating the mean directly in the original scale. Instead the mean has been calculated in the transformed scale and then transformed back into the original scale. The differences between the figures given in Table 1 and the means calculated in the original scale varied between 1 and 3 p.c.; these differences may be ignored.

The number of nymphs which emerged from eggs which remained for 63 days at the alternating temperatures was significantly lower than the number which emerged from the eggs which remained at the alternating temperatures for 53 days. This was true not only of the two "stages" taken as a whole, but also of all the durations (30, 45 and 60 days) at each stage, and also all the temperatures (except 6·5° C.) at each stage. The longer exposure (63 days) to the alternating temperatures had permitted an abnormal growth of the embryo. These eggs were therefore in an abnormal or "unhealthy" condition when they were put away at the constant low temperature. Consequently the elimination of diapause by exposure to low temperature is best studied with reference to those eggs which had had the shorter exposure to alternating temperatures (i.e. stage A of Table 1).

TABLE 1.

*Showing percentage emergence from eggs of* A. cruciata *at 25° C. after they had been exposed to low temperature.*

| | Stage A. Alternating temperature for 53 days. | | | | Stage B. Alternating temperature for 63 days. | | | | Mean (stages A and B). |
|---|---|---|---|---|---|---|---|---|---|
| Temperature. | 30 days. | 45 days. | 60 days. | Mean. | 30 days. | 45 days. | 60 days. | Mean. | |
| 6·5 | 9·6 | 19·4 | 23·2 | 16·9 | 6·4 | 17·2 | 12·6 | 11·6 | 14·2 |
| 10·0 | 42·3 | 51·2 | 66·8 | 54·0 | 26·8 | 36·0 | 34·3 | 32·2 | 43·0 |
| 13·3 | 63·0 | 77·1 | 82·0 | 74·2 | 29·2 | 38·4 | 26·2 | 31·3 | 53·1 |
| Mean | 37·0 | 48·9 | 56·1 | 47·6 | 19·6 | 29·9 | 23·7 | 24·3 | 35·5 |

Significant differences cannot be given directly in terms of per cent. emergence. They must be considered in the transformed scale $\sin^{-1}\sqrt{P/100}$ (where P is the per cent. emergence). In the transformed scale significant differences at the 1 p.c. probability level are:

| | |
|---|---|
| n = 5 (between individual treatments) | 14·2° |
| n = 15 (between temperatures or durations at either stage A or stage B) | 8·2° |
| n = 30 (between temperatures or durations considering both stages together) | 5·8° |
| n = 45 (between stages) | 4·7° |

The per cent. emergence at 13·3° C. (74·2 p.c.) was significantly greater than that at 10·0° C. (54·0 p.c.), and that at 10·0° C. was significantly higher than that at 6·5° C. (16·9 p.c.). The per cent. emergence from the eggs which had remained at the various low temperatures

[2] It was noticed that these eggs were more variable than those of the eastern race with respect to the amount of development which occurred during the exposure to alternating temperatures.

for 60 days (56·1 p.c.) was significantly higher than that for those which had been exposed for 30 days (37·0 p.c.), but it was not significantly greater than that from the eggs which had been exposed for 45 days. The difference (11·9 p.c.) between eggs which had remained at the low temperatures for 45 days and 30 days was barely significant. When individual treatments were considered separately, it was found that the per cent. emergence after exposure to 6·5° C. was significantly lower than that after exposure to 10·0° C. and 13·3° C. for every duration. The per cent. emergence after exposure to 13·3° C. for 45 days was significantly greater than that after exposure to 10·0° C. for the 45 days, but at the other durations (30 and 60 days), these two temperatures did not produce results that were significantly different.

Interaction between the variables in this experiment (i.e. stage, temperature, and duration) can be considered reasonably only in terms of relative increases in per cent. emergence and not in terms of absolute increases. However, the data could not be suitably transformed to logs. for the purpose of making an analysis of variance. Consequently the significance of interaction was not calculated; but the influence of interaction has been deduced from inspection of Tables 2 and 3, in which the data from Table 1 have been rearranged to show interaction.

The figures in Table 2 indicate interaction between temperature and duration. That is, the longer exposure to constant low temperature produced a relatively greater increase in per cent. emergence at the lower temperature than it did at the higher. This is, of course, the expected result since diapause was eliminated more effectively at the higher temperature and once diapause has been eliminated further exposure to low temperature should have no further effect.

TABLE 2.

*Showing interaction between temperature and duration (data for stage A only).*

| Temperature °C. | Ratio of per cent emergence after 30 days to per cent. emergence after | |
|---|---|---|
| | 45 days. | 60 days. |
| 6·5 | 2·00 | 2·30 |
| 10·0 | 1·15 | 1·47 |
| 13·3 | 1·24 | 1·32 |

TABLE 3.

*Showing interaction between temperature and stage.*

| Temperature °C. | Ratio of per cent. emergence of stage B to per cent. emergence of stage A. |
|---|---|
| 6·5 | 1·37 |
| 10·0 | 1·63 |
| 13·3 | 2·30 |

The figures in Table 3 indicate an interaction between temperature and stage, that is the higher temperatures were relatively more effective at stage A than at stage B. This means that the eggs which had been exposed for 63 days to the alternating temperatures were less able to take advantage of the exposure to the more favourable temperatures for the elimination of diapause than were those which had experienced the shorter exposure. This result confirms the deduction drawn from Table 1 that the embryos in the eggs which had been at the alternating temperatures for 63 days had developed abnormally during this long period and were "unhealthy" when the exposure to low temperature began.

## DISCUSSION.

It is clear that the race of *A. cruciata* which occurs in Western Australia resembles that from South Australia and New South Wales in that the eggs enter a state of diapause shortly after they are laid. Diapause was eliminated from the eggs when they were exposed to adequate low temperatures for adequate periods when the embryo was at a suitable stage of development. In order to reach this stage of development the embryo required to be exposed to intermittent low and high temperatures. If this treatment were continued too long the embryo grew beyond the stage at which exposure to low temperature was most effective for eliminating diapause. In all these details the eggs of the race of *A. cruciata* from Western Australia resemble the eggs of the eastern race (Andrewartha, 1943).

The two races differ in that an exposure to 13·3°C. was more effective for eliminating diapause from the eggs of the western race than either 10·0°C. or 6·5°C. whereas with the eastern race 10·0°C. was more effective than either 13·3°C. or 6·5°C.: the last two temperatures were about equal; but 6·5°C. was slightly better than 13·3°C. This relationship is illustrated in Fig. 1. The curves show the per cent. emergence from eggs which had received the most favorable treatment for the elimination of diapause. For the eastern race the eggs had been at alternating temperatures (25° C. and 10° C.) for 40 days and then had been exposed to constant temperature for 60 days. With the western race the eggs had experienced alternating temperatures (25° C. and 13·3° C.) for 53 days, and then had been exposed to constant temperature for 60 days. During the exposure to alternating temperatures the embryos of the eastern race developed uniformly; at the end of 40 days they were all very much at the same stage of development. The embryos of the western race developed less uniformly; so that after 53 days at alternating temperatures they were not so closely grouped around the one stage of development. Thus the former responded more uniformly to the influence of low temperature, giving an emergence of 100 p.c. after an exposure to the most favorable temperature (10°C.), whereas the latter responded less uniformly, giving a maximum emergence of 81 p.c. after exposure to the most favourable temperature (13·3°C.). The figures for the eastern race were taken from results published elsewhere (Andrewartha, 1943).

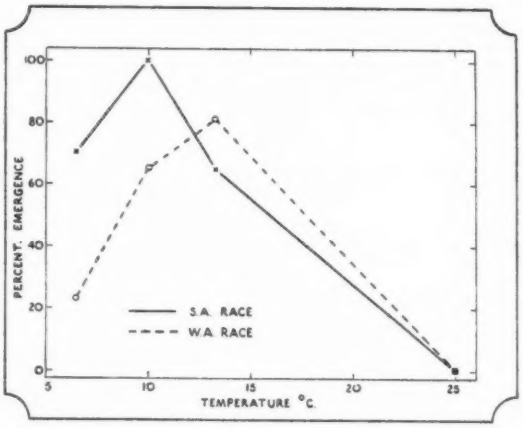


**Fig. 1.** Showing the percentage emergence from eggs of two races of *A cruciata* when they were incubated at 25° C. after being exposed to constant low temperature for the elimination of diapause.

The results of this experiment confirm the conclusions reached from a study of the influence of temperature on the elimination of diapause from the eggs of the eastern race of *A. cruciata*, namely, that diapause is causally related to the nutrition of the embryo and that the elimination of diapause is brought about by a modification of the yolk. The changes in the yolk which go on during the exposure to low temperature convert the yolk into a suitable food for the embryo and also change the physical condition of the yolk so that it no longer obstructs katatrepsis (Andrewartha, 1943).

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# THE ACTION OF METALS ON BACTERIAL INTOXICATION

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Recently Petherick and Singer (1943) described an investigation of the detoxication of diphtheria toxin by metals. In guinea pigs iron, copper or zinc, injected simultaneously with a skin dose of diphtheria toxin, abolished the skin reaction. Manganese, nickel and cobalt were inactive. This detoxication was reproduced *in vitro* by incubating diphtheria toxin in high dilution with iron, copper or zinc compounds together with redox substances such as ascorbic acid, adrenaline or glutathione. It was suggested that detoxication of toxin in the *in vivo* experiments was due to a similar mechanism, the tissue apparently furnishing the redox substances necessary for detoxication.

This paper deals with the effect of the same metals on staphylococcal toxin and the toxins of *Cl. welchii* A and D. Preliminary experiments showed that staphylococcal toxin, injected intradermally into the guinea-pig skin produces reactions bearing a superficial similarity to those caused by diphtheria toxin. Welchii toxins A and D do not produce well-defined skin reactions. Using the method applied with diphtheria toxin, the metal compounds had no effect on the skin reaction produced by staphylococcal toxin.

Accordingly a different technique was adopted and the curative effect of the metals on mice injected with a fatal dose of toxin was investigated.

## CURATIVE ACTION OF METALS.

*Material and methods.* White mice, bred at the Institute, were used throughout. They averaged 20 gm. weight and were kept on a diet of bread, bran, bone meal, sunflower seeds and greens.

The metal compounds used were A.R. reagents with the exception of ferricitrate B.P.

Injections of metals and toxins were made subcutaneously into different sites.

Diphtheria toxin, staphylococcal toxin and welchii A toxin were kindly supplied by the Commonwealth Serum Laboratories and purified welchii D toxin by Dr. Turner of the C.S.I.R.

*Method of treatment.* As a preliminary, the doses of the different metal compounds which were fatal to mice when given in a single injection were determined.

TABLE 1.

*Fatal doses of metal compounds expressed as mg. metal per 20 gm. mouse.*

| Compound. | Fatal dose. |
|---|---|
| $FeSO_4.7H_2O$ | 1·0 mg. |
| Ferricitrate | 0·5 mg. |
| $CuSO_4$ | 0·6 mg. |
| $NiSO_4$ | 0·5 mg. |
| $CoSO_4$ | 1·0 mg. |
| $MnSO_4$ | 2·0 mg. |
| $ZnSO_4$ | 2·0 mg. |

In the experiments with staphylococcal and welchii A and D toxins, the following treatment was adopted:

[1] Working under a full-time grant from the National Health and Medical Research Council.

The mice were given, over a period of 2 days, 4 injections of one-tenth the fatal dose of the metal. On the morning of the third day a fatal dose of toxin was injected followed one hour later by a fifth injection of metal compound. The total amount of metal administered was, therefore, one-half the dose fatal if given in a single injection.

As it is difficult to produce fatal diphtheria intoxication in mice, the treatment was modified to suit the particular conditions of these tests.

An amount of toxin, which would kill all the controls when injected daily over a period of from 10 to 12 days, was used for the experiments with diphtheria toxin and a control experiment with welchii D toxin. Two injections of the metal compounds totalling one-fifth of the fatal dose were given on the day preceding the first injection of toxin. Thereafter one injection of one-twentieth of a fatal dose of the metal compound, followed after 3 hours by an injection of toxin, were given daily until the death of the last control.

Survival for 3 days after the death of the last control was accepted as the end point of the experiment since very few delayed deaths occurred in such experiments. These are mostly due to intercurrent infections precipitated by the toxaemia.

TABLE 2.

*Curative experiments on mice injected with fatal dose of toxin.*

Figures in italics give number of survivors, figures in Roman type give number of deaths.

| | | Metal compounds used for injection and total amount of metal administered in mg. per 20 gm. mouse. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Toxin. | Fatal doses of toxin. | $FeSO_4$ 0·5. | | Ferri-citrate 0·4. | | $CuSO_4$ 0·5. | | $ZnSO_4$ 2·0. | | $CoSO_4$ 0·5. | | $NiSO_4$ 0·25. | | $MnSO_4$ 1·0. | | Controls. | |
| Staphylococcal | 0·045 ml. | *7* | 9 | *4* | 8 | *4* | 8 | *4* | 4 | *2* | 6 | *2* | 6 | *7* | 1 | *1* | 19 |
| Welchii A | 0·18 ml. | *10* | 2 | *4* | 4 | *5* | 3 | *6* | 2 | *6* | 2 | *6* | 2 | *5* | 3 | *2* | 14 |
| Welchii D | 0·075 mg. | *1* | 7 | — | | *1* | 6 | *4* | 4 | *0* | 7 | *2* | 6 | *4* | 4 | *1* | 11 |
| Diphtheria | 12 × 0·1 ml. | *6* | 1 | — | | *3* | 5 | *2* | 6 | *0* | 8 | *2* | 6 | *2* | 6 | *0* | 8 |
| Welchii D | 10 × 0·0125 mg. | *0* | 4 | — | | *0* | 4 | *2* | 2 | *0* | 4 | *2* | 2 | *2* | 2 | *0* | 4 |

Only simple metal compounds were used in these tests. As these caused severe infiltration and, in some cases, necrosis, it was necessary to determine whether the curative action was due to a specific effect of the metal or to a non-specific increase of resistance. In further experiments the metals were administered in the drinking water. The greatest amount that could be administered in this way was limited by the concentration that the mice would accept without undue restriction of the fluid intake. As the average daily consumption of fluid per mouse was approximately 2 c.c., the amount of metal introduced in this way was rather less than that administered by injection. Variations in uptake and irregular absorption from the intestines must be considered.

Administration of metal compounds in the drinking water began 2 days prior to the injection of toxin and was continued throughout the experimental period, which lasted 10 days in the case of staphylococcal, welchii A and D toxins, and 15 days in the experiment with diphtheria toxin. To prevent cumulative toxic effects, the concentration of the metal solution was reduced to one-quarter on the fifth experimental day.

TABLE 3.

*Curative experiments on mice injected with fatal dose of toxin.*

Figures in italics give number of survivors, figures in Roman type give number of deaths.

| | | Metal compounds administered in drinking water and metal content of water at commencement of experiment in mg. per ml. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Toxin. | Fatal doses of Toxin. | Ferricitrate 0·32. | | $CuSO_4$ 0·25. | | $ZnSO_4$ 0·5. | | $CoSO_4$ 0·2. | | $NiSO_4$ 0·1. | | $MnSO_4$ 0·5. | | Controls. | |
| Staphylococcal | 0·04 ml. | *6* | 2 | *2* | 6 | *1* | 7 | *4* | 4 | *5* | 3 | *6* | 2 | *2* | 6 |
| Welchii A | 0·15 ml. | *4* | 3 | *3* | 5 | *4* | 4 | *4* | 3 | *4* | 4 | *5* | 3 | *2* | 6 |
| Diphtheria | 10 × 0·1 ml. | *3* | 3 | *1* | 5 | *2* | 4 | *2* | 4 | *0* | 6 | *2* | 4 | *0* | 6 |
| Welchii D | 0·075 mg. | *0* | 6 | *0* | 6 | *0* | 6 | *1* | 5 | *1* | 5 | *3* | 3 | *0* | 6 |

In judging the results of these experiments, it must be realized that they were designed for only one purpose. It had to be decided whether it was possible to prevent the death of mice injected with a fatal dose of different toxins by the administration of metal compounds. The experiments in which the metals were administered in the drinking water were performed to

obviate the possibility that the increase in resistance observed when metal compounds were injected subcutaneously was caused by a non-specific inflammatory effect. No attempts were made to determine optimal dosage or timing of administration. Refinements in experimental technique would have required a much larger number of mice. This was not justified, as the simple compounds tested are unsuited for exact determination of curative action.

The results with the two methods of administration are similar. Much lower doses of the metals may have been effective when given by injection.

Diphtheria intoxication is strongly influenced by iron in the form of ferrous sulphate, and moderately by ferricitrate and copper. The effect of zinc, nickel, cobalt and manganese is doubtful.

The results of the protection experiments on mice are practically identical with those obtained in the guinea-pig skin and with *in vitro* methods. The metals which were able to prevent the skin reaction or to detoxify the toxin *in vitro* could prevent the death of mice injected with a fatal dose of the toxin. Zinc, which had a slight effect only when tested on the guinea-pig skin or *in vitro*, was almost inactive in experiments on mice as were the metals which were inactive in the guinea-pig or with *in vitro* tests.

Experiments with the other toxins showed that iron and manganese were very active with staphylococcal toxin, zinc and copper were slightly active, and cobalt and nickel were inactive. Ferrous sulphate was very active with welchii A toxin and all the other metals had a marked, but lesser, curative effect. Striking differences were observed in the experiments with welchii D toxin. Only manganese was active in both types of experiment, zinc was active to a moderate extent when injected but was inactive when administered in the drinking water, and all the other metals were inactive.

The fact that the activity of the different metals varied with the toxin attracts attention, especially the effect of manganese on intoxication with staphylococcal, welchii A and D toxins. This makes the ambiguous results with diphtheria toxin rather more significant.

The strong activity of nickel and cobalt against welchii A toxin and their slight activity in some other intoxications suggest that the clear cut results which were obtained in the experiments on the guinea-pig skin and *in vitro* indicate only one of the possible mechanisms of detoxication in which biologically active metal compounds are implicated. The activity of the metals varies apparently not only with the toxin but with the organ affected. In experiments with diphtheria toxin, iron, copper and zinc are active in the guinea-pig skin, whereas manganese is devoid of activity but has some slight activity in generalized intoxication of mice. This fact could be explained by assuming that manganese cannot be activated in the skin but by some other organ.

To elucidate this point the effect of differences in the properties of the metals and the toxins was investigated.

Two fundamentals had to be disregarded, namely, the differences in the chemical constitutions of the toxins and in the distribution and chemical combinations of the different metals in the body of the experimental animals. Investigation of these questions would lead into fields of such complexity and difficulty that chances of success would be practically non-existent. If one accepts these restrictions only two problems remain, the solution of which could contribute to an understanding of the observed results:

(1) What are the differences between the reaction of the different toxins and the animal body which cause the varying activity of the metals?

(2) By what substances are manganese, nickel and cobalt activated as iron, copper and zinc are activated by ascorbic acid, adrenaline or glutathione?

For technical reasons the experiments were restricted to welchii D and staphylococcal toxins and manganese.

## AFFINITY OF TOXINS FOR GUINEA-PIG SKIN.

In experiments with skin doses of staphylococcal toxin, the first signs of the reaction were observed after from 3 to 4 hours. In analogous experiments with diphtheria toxin, the reaction appeared after 24 to 48 hours. The rapid action of staphylococcal toxin might explain our failure to influence the skin reaction by the simultaneous injection of metal compounds.

The fate of intradermally injected toxins was investigated.

*Method:*

*Diphtheria toxin and staphylococcal toxin.* Several intradermal injections, each containing 20 skin doses of toxin in a volume of 0·1 ml., were given at varying intervals. The animal was then killed and the cutis excised, finely minced, mixed with saline, frozen, thawed three times and centrifuged. The total volume of tissue and extraction fluid was adjusted to 1 ml. If all the toxin were recovered, 0·1 ml. of the extract would contain 2 skin doses. This amount was injected intradermally into a guinea-pig.

*Welchii D toxin.* This toxin did not produce skin reaction. Ten fatal mouse doses were injected in a volume of 0·2 ml., the same technique was used for preparing the extracts, and 0·2 ml. was injected subcutaneously per 20 gm. mouse, 2 mice being used for each test. If all the toxin were recovered this volume would contain 2 fatal doses.

The results of these experiments are set out in Table 4.

TABLE 4.

| Time after injection. (min.). | Toxin. Diphtheria. | Staphylococcal. | Welchii D. |
|---|---|---|---|
| 10 | + | — | ++ |
| 30 | + | — | +0 |
| 60 | + | — | +0 |
| 2 hours | + | — | 0 0 |
| 4 „ | + | — | 0 0 |

Diphtheria toxin and staphylococcal toxin: + Reaction equivalent to that produced by one skin dose. — No reaction.

Welchii toxin: ++ Both mice dead. +0 One mouse dead. 0 0 No mice dead.

These experiments show that the affinity of diphtheria toxin for the skin of the guinea-pig is very low as toxin was recovered up to 4 hours after injection. In comparable experiments with staphylococcal toxin, all toxin had disappeared within 10 minutes. The reaction of staphylococcal toxin with the guinea-pig skin is, therefore, instantaneous, because the wheal raised by the intradermal injection is clearly visible for 5 minutes, and during this time contact between toxin and cells must be poor.

Welchii toxin was demonstrated up to 1 hour after the injection of 10 fatal doses. However, the experiments with welchii D toxin are not strictly comparable with those with diphtheria and staphylococcal toxins.

Skin doses were used in the experiments with diphtheria and staphylococcal toxins and fatal doses for mice in the experiments with welchii D. The amount of toxin-protein injected is not known but it is probable that the fatal dose for mice contains a larger amount of toxin-protein than that necessary to produce a local skin reaction in the guinea-pig. It is probable that, in the experiments with welchii D toxin, the amount of toxin injected was excessive and could not be absorbed therefore as quickly as the small amounts of staphylococcal toxin.

One is justified in concluding that both staphylococcal and welchii toxins have a much greater affinity for guinea-pig skin than has diphtheria toxin.

## DETOXICATION OF DIPHTHERIA TOXIN BY MANGANESE.

The technique applied in the investigation of the activity of iron, copper and zinc was used in these experiments. High dilutions of diphtheria toxin in phosphate buffer or inactivated rabbit serum were exposed at room temperature and 37° C., to the action of various combinations of manganese and other substances. The effect on the toxin was then tested by intradermal injection of appropriate dilutions into guinea-pigs.

A considerable number of possibilities had to be investigated and a short list of combinations which were found to be inactive is given below. The reasons which prompted the various investigations are given at the beginning of each series.

1. Increasing the detoxifying effect of iron, copper and zinc plus redox substances:
   (i) Mixtures of toxin, manganese and iron, cytochrome c, copper or zinc, plus ascorbic acid, adrenaline or glutathione in phosphate buffer or inactivated rabbit serum.
   (ii) Mixtures of toxin, manganese and iron or cytochrome plus cysteine in inactivated serum.
2. Stimulating the production of ascorbic acid by manganese:
   Mixtures of toxin and manganese with glucose, galactose or mannose in phosphate buffer, inactivated rabbit serum and extracts from mouse and guinea-pig organs (Rudra, 1939a and b).

3. Activation of manganese by complement:
   Mixtures of toxin and manganese with and without addition of cysteine in active rabbit serum.
4. Specific activation of manganese by vitamin $B_1$.
   Mixtures of toxin and manganese with vitamin $B_1$ in phosphate buffer (Foster *et al.*, 1942; Perla, 1939).
5. Activation of proteolytic enzymes by manganese:
   Mixtures of toxin and manganese with organ extracts of mouse and guinea-pig with and without addition of cysteine.

The only experiments which gave positive results are discussed below. Hydrogen peroxide activated the detoxication of diphtheria toxin by manganese. Dilutions of diphtheria toxin in Sörensen's phosphate buffer of pH 6·6 were set up in test tubes and the appropriate additions of manganese, as manganese sulphate, and hydrogen peroxide were made.
The total volume was 3 ml., the toxin content being 30 skin doses per 0·1 ml. After incubation at 37° C. for 5 hours, 0·1 ml. of a 1/30 dilution of each mixture was injected into guinea-pigs. The results are given in Table 5.

Slight variations in results occurred, hence every experiment is recorded, the figures indicating the number of tests made, e.g. with a concentration of hydrogen peroxide of $1 \times 10^{-6}$ and no addition of manganese, in six different experiments the intradermal reaction following injection of the toxin dilution was ++. This is given in the Table as 6++.

TABLE 5.

| Compound tested. | Metal mg. p.c. | Concentration of $H_2O_2$. | Skin reaction. |
|---|---|---|---|
| — | — | $1 \times 10^{-5}$ | 6++ |
| | | $5 \times 10^{-5}$ | 1++ |
| | | $1 \times 10^{-5}$ | 1++ |
| | | $5 \times 10^{-5}$ | 1++ |
| | | $2{\cdot}5 \times 10^{-5}$ | 1++, 1± |
| $MnSO_4$ | 1·5 | $1 \times 10^{-5}$ | 3++, 2+, 1± |
| | | $5 \times 10^{-5}$ | 1++ |
| | | $1 \times 10^{-5}$ | 1 0 |
| | | $5 \times 10^{-4}$ | 1± |
| | | $2{\cdot}5 \times 10^{-4}$ | 2 0 |

++ Signifies the reaction obtained with one skin dose of diphtheria toxin consisting of redness and infiltration one cm. in diameter. +, ± Reactions weaker than one skin dose. 0 No reaction.

The effect of mixtures of other metals with hydrogen peroxide was then investigated. Detoxication occurred when toxin was incubated with iron, copper or zinc and hydrogen peroxide. Cobalt and nickel were not activated. The results with iron, copper and zinc are shown in Table 6.

TABLE 6.

| Compound tested. | Metal mg. p.c. | Concentration of $H_2O_2$. | Skin reaction. |
|---|---|---|---|
| $CuSO_4$ | 1·5 | $1 \times 10^{-5}$ | 2++, 2±, 1 0 |
| | | $5 \times 10^{-5}$ | 1 0 |
| | | $1 \times 10^{-5}$ | 1 0 |
| | | $5 \times 10^{-4}$ | 1 0 |
| | | $2{\cdot}5 \times 10^{-4}$ | 1 0 |
| $ZnSO_4$ | 1·5 | $1 \times 10^{-5}$ | 1++, 3± |
| | | $2{\cdot}5 \times 10^{-4}$ | 1 0 |
| $FeSO_4$ | 1·5 | $1 \times 10^{-5}$ | 1++, 3± |
| | | $2{\cdot}5 \times 10^{-4}$ | 1 0 |

The controls given in Table 5 apply to both series of experiments.

Iron, copper and zinc are effective with both redox substances and hydrogen peroxide as activators. The question arises whether hydrogen peroxide is the direct activator of the detoxication of toxin by these metals. The aerobic oxidation of the redox substances and the production of hydrogen peroxide would thus be the first step in a two stage reaction. The second step would then be the oxidation of the toxin molecule by the combined action of hydrogen peroxide and metal compounds. This is a possibility which deserves serious consideration.

The active concentrations of redox substances and hydrogen peroxide are in fair agreement. The different activities of iron, copper and zinc on the one hand and manganese on the other could be explained by different distribution of these metals in the animal body. Recent biochemical investigations indicate that hydrogen peroxide has greater importance in metabolic processes than has been ascribed to it previously. (Abrams *et al.*, 1942; Altschul *et al.*, 1940; Huszak, 1942).

According to these investigators hydrogen peroxide is an important metabolite and should not be considered as a noxious by-product of oxidative metabolism, which is at once destroyed by catalase.

Facile acceptance of this hypothesis is unwarranted, because knowledge of oxidative processes outside of carbohydrate metabolism is extremely scanty, and not applicable to the problem of detoxication of toxins.

## DISCUSSION.

In these studies on diphtheria, staphylococcal and welchii A and D toxins a detoxication mechanism has been demonstrated which operates by the combined action of metal compounds and either redox substances or hydrogen peroxide.

A number of iron compounds has been tested with diphtheria toxin, and those which contained iron in the ionized form showed the highest activity. Metal-protein-combinations may be more active than inorganic salts, as inorganic salts are more active when dissolved in serum than when dissolved in broth or saline.

The detoxication might be an oxidative process, the toxin molecule being directly oxidized by activated oxygen, the components of the detoxication system varying with the different toxins. Iron, copper and zinc and ascorbic acid, glutathione and adrenaline are most active with diphtheria toxin, experiments with the guinea-pig skin, general intoxication of mice, and *in vitro* tests giving identical results.

The toxin of welchii D, however, is resistant to this mechanism, but is subject to detoxication by a system consisting of manganese plus hydrogen peroxide; staphylococcal and welchii A toxin can be detoxified by both. Iron is more active in an intoxication in which the toxin reacts slowly with the tissues; manganese is more active where the toxin reacts more quickly. These differences recall the results of Walbum (1925-26) who tested systematically the influence of nearly all the elements of the periodic system in a variety of bacteriological and serological problems. He found manganese active in anti-toxin production, beryllium in agglutinin production, caesium and iridium in mouse typhoid infections, and aluminium, manganese and gold in guinea-pig tuberculosis. In intoxication of rabbits with staphylococcal, diphtheria or dysentery toxin, best results were obtained with manganese. Walbum could not give an explanation of these results but considered activation of enzymes and increase in antibody production. According to our results this explanation seems to be erroneous, but the general conclusions that different metals are active in different infections and toxic states are confirmed by our observations.

As toxin is detoxified in the organs and not in the blood, and as iron in the form of cytochrome was more active than in the form of haemoglobin or ferritine, it appears that the metal proteins which determine the resistance against infectious diseases and intoxications are constituents of organ cells.

Investigations will have to be undertaken to determine the metallo-protein or proteins which are most severely affected in the course of infectious diseases. Iron alone will probably not be effective in the maintenance of resistance to infectious diseases as it has been shown that iron, by itself, is insufficient to restore the normal enzymatic activity of depleted organs. (Cohen and Elvehjem, 1934; Frost, Spitzer, Elvehjem and Hart, 1941; Schultze, 1939; Whipple, 1942).

The activity of the metals is not restricted to bacterial intoxication but extends to other toxaemic states. It has long been known that anaphylactic shock can be prevented by manganese, ascorbic acid, sodium thiosulphate or cysteine (Hochwald, 1936; Klopstock, 1925).

In preliminary experiments it was found that iron can prevent local anaphylactic reactions in the guinea-pig skin, that iron and copper reduce the intradermal tuberculin reaction in tuberculous cows, and that iron and manganese reduce the reaction following a Mantoux test in humans.

These experiments indicate that the detoxication mechanism which has been discussed is active in a very wide range of toxaemic conditions and furnish a rational basis for the well-known fact that resistance against infectious diseases is lowered by malnutrition.

## SUMMARY.

The detoxication of diphtheria, staphylococcal and welchii A and D toxins has been investigated.

Two detoxication mechanisms have been described. In the first, iron, copper or zinc and ascorbic acid, adrenaline or glutathione are implicated. The second operates by the combined action of manganese and hydrogen peroxide.

The theoretical importance of these observations has been discussed.

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# RED CELL AGGLUTINATION BY VACCINIA VIRUS

## APPLICATION TO A COMPARATIVE STUDY OF VACCINATION WITH EGG VACCINE AND STANDARD CALF LYMPH

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In a previous paper (Nagler, 1942), it was shown that Hirst's phenomenon could be applied to the titration of vaccinia virus and of antibody provoked by vaccination in human subjects, or in experimental animals. The methods then developed have now been applied to some of the practical problems of human vaccination against smallpox. Investigations have been made to compare the antibody responses following vaccination with standard calf lymph and with vaccinia virus grown according to Goodpasture's method on the chorioallantois of the chick.

Two different types of "egg vaccine" were used. The first was prepared from membranes sown with diluted calf lymph so that first egg passage material was used for the actual vaccinations. It was assumed that no recognizable modification would have taken place by such a single passage. The second type of vaccine had undergone 20 passages on the chorioallantois and might therefore have undergone a certain amount of modification.

Incidental to this work a number of observations have been made on the nature of the antibody response in relation to the initial status of the subject (unvaccinated or vaccinated) and to the type of local reaction produced.

During the period covered by this investigation (1941-1942) there was an unusually active demand for vaccination in Melbourne, and through the co-operation of the Free Kindergarten Union of Victoria large numbers of children were vaccinated with one or other of the different batches of egg vaccine or calf lymph. In only a small proportion of these were serum tests made, but the clinical results observed in these groups are reported as a contribution toward assessing the value of this type of vaccine. With the same object in view the results of comparative determinations of the keeping qualities of calf lymph and egg vaccine are also included.

### MATERIALS.

I am indebted to the Commonwealth Serum Laboratories for calf lymph Nos. 3993 and 4076 (glycerolated) and further samples of unglycerolated calf lymph for experimental work.

The egg vaccine B1 was prepared in this laboratory and represents the first egg passage of the original calf lymph 4076. The egg vaccine A20 used in the third series of vaccinations represents the 20th egg passage of calf lymph C.S.L. 3993 from the same Commonwealth Serum Laboratories stock vaccine as No. 4076.

The standard immune-serum V2 used throughout this work was obtained from a rabbit inoculated with two intracutaneous injections of virus suspension at eight days' interval. The animal was bled twenty days after vaccination, the serum removed from the clot and inactivated at 56° C. for 20 minutes.

Human sera were obtained from subjects vaccinated either with calf lymph or with egg-membrane vaccine. The subjects were bled immediately before and 21 days after vaccination.

Red blood corpuscles were obtained from blood taken from the wing vein of a fowl into citrate saline solution. The cells were washed twice and made up to a 2 p.c. suspension in physiological saline or calcium saline solution. It has been found that cells from a number of fowls were only slightly or not at all susceptible to the agglutination effect of vaccinia virus. A paper partly concerned with that fact is in preparation. Each batch of cells should therefore be checked with a virus suspension known to be active.

## METHODS.

*Vaccinia virus suspension for vaccination* was produced as follows: Stock virus, usually diluted 1: 100 in broth, was inoculated by Burnet's method (Burnet, 1936) on to the chorioallantoic membrane of 10 or 12-day chick embryos. After three days' further incubation the chorioallantoic membranes which showed a confluent mass of specific foci with secondary oedema and cellular infiltration, were cut out. The suspension of virus was then prepared by grinding these membranes with quartz powder in a mortar under as sterile conditions as possible. The material was taken up from the mortar with 0·5 c.c. of glycerol-Ringer solution per membrane and then spun down for 15 minutes at 2,500 r.p.m. A second spinning was then performed with the supernatant fluid for 20 minutes at 15,000 r.p.m. in order to clarify the material as much as possible.

Tests on the chorioallantoic membrane of chick embryos were carried out to determine the virus content of suspensions before and after high speed centrifugation for 20 minutes, and it was found that there was no apparent decrease of living virus particles in those suspensions.

Sterility tests carried out aerobically and anaerobically on several samples always showed that the materials concerned were free from bacterial contamination.

*Vaccinia virus for the agglutination test* was produced in a similar manner. After removal from the eggs the membranes were kept under sterile conditions in the ice chest for further two days. This facilitates the procedure of grinding. The material was taken up from the mortar with normal saline solution, containing 0·044 p.c. $CaCl_2$, allowing 0·5 c.c. per membrane. After spinning at 2,500 r.p.m. the supernatant fluid was used as the virus suspension in the agglutination tests.

In the course of the investigations it was also found possible to use glycerolated Ringer solution for suspending the ground membranes without loss of activity. This applied especially to tests where it was thought advisable to use a standardized material throughout larger series.

*The estimation of the activity of vaccines* was carried out on the chorioallantoic membrane by Burnet's method. The materials were prepared in dilutions of $10^{-4}$, $10^{-6}$, $10^{-8}$, $10^{-7}$ and $10^{-9}$ and 0·05 c.c. of each dilution inoculated on the chorioallantoic membrane of four hen eggs which had been incubated for twelve days. After a further 72 hours' incubation the membranes were excised and the number of foci counted on each membrane.

*Method of vaccination.* Vaccination was performed by the "multi-pressure" method, sponsored by the U.S. Public Health Service. The skin over the insertion of the deltoid in the left upper arm was cleaned gently with ether and allowed to dry. A small drop of vaccine was placed on the cleaned area and 15–20 pressures were made with a sterile needle held parallel to the skin. The needle was pressed downward into the skin so that at the tip there was a minute break in the epidermis without bleeding. After two minutes the excess of vaccine was gently wiped off. No dressing was applied in case of the children from kindergarten schools. Subjects vaccinated in this Institute were provided with a light gauze pad fixed with adhesive plaster over the site of vaccination.

The result of the vaccinations were recorded in the manner recommended by Leake as follows:

1. Primary reaction; this developed slowly, giving a maximum development of erythema in from 8–14 days with often considerable constitutional symptoms for a day or two at that time. The vesicle developed in 3–5 days and spread outward till a day or two after the maximum area of erythema had developed.

2. Accelerated reaction, indicating partial immunity, showed maximum erythema in 3–7 days with definite vesicle formation but rapid drying of the crust and very superficial scar or none at all.

3. Immune reaction took the form of a narrow zone of erythema reaching a maximum in 8–72 hours and was nearly always associated with itching but no vesicle formation.

Complete lack of reaction on the skin was recorded as a "non-take" and was regarded as a fault in the technique of inoculation or in the quality of lymph.

*Method of antibody titration.* In each series of tests the virus suspension was titrated first against the red cells. The virus suspension was distributed in falling dilutions of 1/2, 1/4, 1/8, etc. in 0·25 c.c. amounts. The tubes used had an inner diameter of approximately 1·0 cm. An equal amount of 2 p.c. suspension of chick or hen cells was added and the fluid in each tube was made up to 0·75 c.c. with saline solution. After shaking, the mixtures were allowed to stand in the incubator for one hour at 37° C. and then examined. The results were recorded +++, ++, +, ±, 0. (+++ represents maximal agglutination, in which the cells are distributed in compact clumps that adhere to the glass over most of the bottom of the tube; ++ means a nearly complete agglutination, somewhat finer in surface appearance and more concentrated at the bottom of the tube; + indicates an incomplete agglutination with an unagglutinated centre; ± indicates only a trace of very fine agglutination at the edge of the unagglutinated deposit. In tubes in which no agglutination occurs (0) the cells settle to a small uniform deposit with a smooth circular edge at the bottom of the tube.)

The human sera were titrated as follows: falling quantities of serum (1/2, 1/5, 1/10, 1/20, 1/50, etc.) in a volume of 0·25 c.c. were mixed with a constant amount of virus in similar volume.

The virus anti-serum mixture was allowed to stand for half an hour in the incubator at 37° C. before the final addition of red cell suspension. The results were read after incubation for a further hour.

The virus suspension used for each batch of titrations was standardized against the immune rabbit serum V2. As a rule stock virus diluted 1: 100 was completely inactive when mixed with standard serum 1: 20 and produced a distinct, weak agglutination (±) with serum 1: 50. If necessary the dilution of the virus was adjusted to more or less than 1: 100 to give the standard ± reaction with 1: 50 serum.

The titre of each human serum was estimated from the tube showing ± agglutination, intermediate values being interpolated if necessary. The neutralizing power of the standard serum was taken as 100 units. When a human serum diluted 1: x and mixed with virus gave the same ± reaction as the standard serum diluted 1: 50, then the titre of the human serum was 2 x units. An example of such a titration is shown in Table 1.

**TABLE 1.**

*Protocol of typical antibody titration.*

| | Dilution of standard and human serum. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1/2 | 1/5 | 1/10 | 1/20 | 1/50 | 1/100 | 1/200 | 1/500 |
| Standard serum V2 | 0 | 0 | 0 | 0 | ± | ++ | +++ | +++ |
| Human serum No. 28, before vaccination | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Human serum No. 28, three weeks after vaccination | 0 | 0 | 0 | 0 | 0 | ± | ++ | +++ |

Each tube contained in addition to the serum dilution equal amounts (0·25 c.c.) of a 1/100 diluted vaccinia virus and 2 p.c. red cell suspension.

*The Antibody Response after Vaccination with Calf Lymph and Egg Vaccine.*

(a) *Primary vaccinations.* In presenting the results of antibody titrations it is convenient to deal first with the results in previously unvaccinated subjects, all of whom showed typical primary-type lesions. As in all the tests to be reported blood samples were taken immediately before vaccination and again three weeks later. The sera were separated, inactivated and stored in the refrigerator until it was convenient to titrate them. In all instances the first and second serum samples of each subject were titrated at the same time with the same reagents so as to ensure an accurate comparison.

Twenty-nine subjects in the previously unvaccinated group were vaccinated with calf lymph 4076. All sera taken before vaccination showed a titre of less than 4 units per c.c. Three weeks after vaccination the increase was to 25–45 units in three sera, to 50–95 units in 11 sera, to 100–195 units in 13 sera, and to 200–400 units in 2 sera. The average increase in that group was 103 units.

Twenty-nine subjects vaccinated with egg vaccine B1 have been similarly tested. All sera of this group taken before vaccination had a titre of less than 4 units per c.c. Three weeks after vaccination the titres observed were 25–45 units in 2 sera, 50–95 in 5 sera, 100–195 in 13 sera and 200–400 in 9 sera; an average increase of 142 units.

A third group of 32 subjects was vaccinated with egg vaccine A20. In this group also the initial sera all showed an antibody content of less than 4 units per c.c. Three weeks after vaccination an increase to 25–45 units was observed in 3 sera, to 50–95 in 15 sera, to 100–195 in 13 sera and to 200–400 in 1 serum; an average increase of 86 units per serum.

In Fig. 1 the antibody response in these groups three weeks after vaccination is shown graphically.

No significant differences could be observed in the clinical appearance of the lesions produced by the three different types of vaccine, nor was there any correlation between the extent of the scar and the height of the antibody response.

(b) *Revaccinations.* In this group are included all subjects who had been vaccinated at any time previously. As would be expected a wide range of the intensity of the lesion produced was observed. Of 58 subjects in the group, 12 gave reactions which were classed as primary reactions, 31 as accelerated reactions and 15 as immune reactions. As in the previous group of unvaccinated persons, the three vaccines, calf lymph 4076 and egg vaccines B1 and A20 were used for 22, 22 and 14 subjects respectively, but analysis of the results showed that the type of clinical response had much more significance in determining the extent of the antibody rise, than the particular vaccine used. In general it was found that about 50 p.c. of the group (30/58) had a detectable amount of antibody in the serum from the initial bleeding, that subjects showing a primary type reaction gave an antibody response equivalent to that observed previously in unvaccinated subjects, while the response became progressively less as the reaction approached the

trivial immune type. Fig. 2 shows clearly the correlation between the type of clinical response and the antibody titres attained three weeks after vaccination. The average final titres for the three groups given the different vaccines were: Calf lymph 4076, 14 units; egg vaccine B1, 29 units; egg vaccine A20, 33 units. These differences should not, however, be taken as significant, as the numbers were too small to allow the results to be analysed adequately both for vaccine type and for the immune status of the subject.

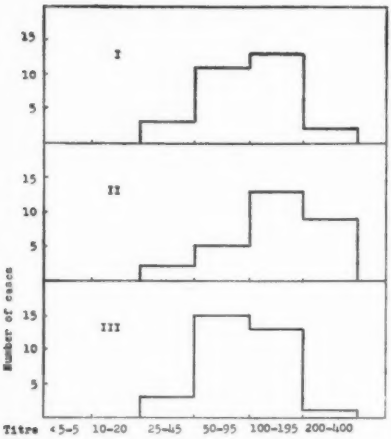


**Fig. 1.** Distribution of antibody titres in previously unvaccinated subjects vaccinated with (I) calf lymph 4076 (29 cases), (II) egg vaccine B1 (29 cases), (III) egg vaccine A20 (32 cases).

The serum samples tested were taken three weeks after vaccination. The ordinates represent the number of subjects showing the antibody titres indicated by the abscissae.

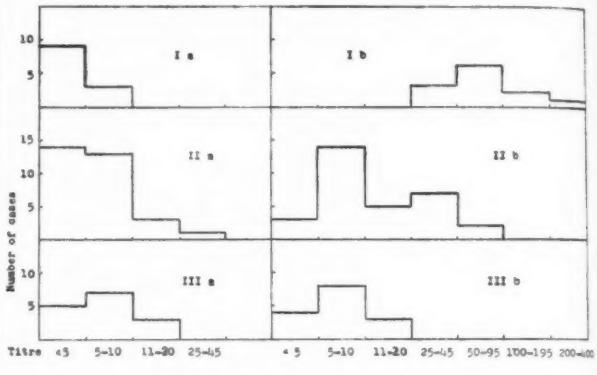


**Fig. 2.** Distribution of antibody titres (a) before and (b) three weeks after vaccination in subjects who had previously been vaccinated. I 12 subjects showing a primary type reaction, II 31 subjects showing accelerated reactions, III 15 subjects showing immune type reactions.

*Relative Effectiveness of Calf Lymph and Egg Vaccine for Large Scale Vaccinations.*

About 1,800 children from 6 months to 12 years were vaccinated with calf lymph or egg vaccine, the standard multiple pressure method being used throughout. The vaccinations were done by several different operators, but all were experienced in the method. The results of vaccinations were recorded as primary, accelerated or immune reactions and failures to take. Primary vaccinations will be considered first. All of these gave either a definite primary reaction or a complete failure to take. No difference in the average intensity of the reactions could be ascribed to the type of vaccine used, and there were no complications or severe secondary infections in any of the groups. Table 2 shows the proportions of failures in the three groups vaccinated with calf lymph 4076, and egg vaccines B1 and A20.

TABLE 2.

*Primary vaccination with calf lymph 4076, egg vaccines B1 and A20.*

| Kindergarten No. | Types of vaccine used for vaccination. | Number of vaccinations. | P.c. of non-takes. |
|---|---|---|---|
| 1–4 | Calf lymph 4076 | 325 | 3·8 |
| 5–11 | Egg vaccine B1 | 612 | 13·1 |
| 12–20 | Egg vaccine A20 | 721 | 0·3 |

In tabulating the results with previously vaccinated children (and a few adults done at the same time) all definite reactions were taken as successful vaccinations. Table 3 shows the proportion of failures with each vaccine.

TABLE 3.

*Re-vaccinations with calf lymph 4076, egg vaccines B1 and A20.*

| Kindergarten No. | Types of vaccine used for vaccination. | Number of vaccinations. | P.c. of non-takes. |
|---|---|---|---|
| 1–4 | Calf lymph 4076 | 47 | 9·4 |
| 5–11 | Egg vaccine B1 | 90 | 34·4 |
| 12–20 | Egg vaccine A20 | 105 | 0 |

It will be seen that in both groups the vaccines are increasingly effective in the order: B1, < calf lymph 4076, < A20. The percentage of failures with the first passage material was considerably higher than with either of the others. This may have been in part due to minor differences in the technique used by different operators as a large proportion of the failures were in two kindergartens, where there were 53 failures in 234 vaccinations (23 p.c.) as contrasted with 27 failures in 378 vaccinations done in five other kindergartens (7·1 p.c.). However, this assumption does not alter the clear evidence that batch A20 was superior to B1 in the proportion of successful takes produced.

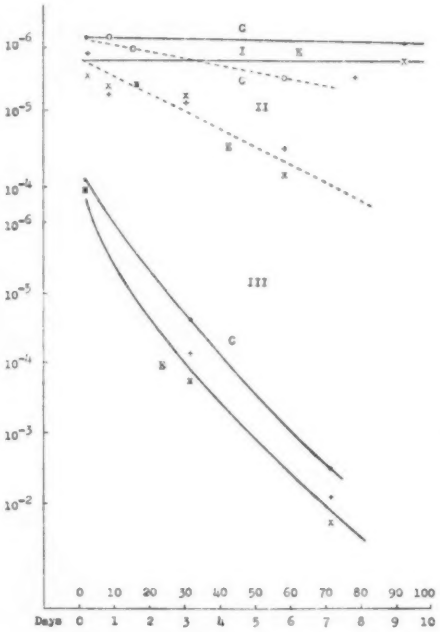


**Fig. 3.** To illustrate comparative keeping qualities of calf lymph and egg vaccine.

Repeated titrations of two samples of glycerolated egg vaccine (E) and one of calf lymph (C) kept I at refrigerator temperature (± 4° C.), II at room temperature in the dark, and III at 37° C.

The titres obtained by chorioallantoic titration are plotted logarithmically, each division representing a tenfold difference. Time is in days, the scale for the 37° C. experiment being ten times that for the other two.

From the graphs the following approximate times for a decrease of titre to one-tenth of the original can be calculated: at refrigerator temperature neither shows significant fall. At room temperature calf lymph 98 days, egg vaccine 40 days. At 37° C. calf lymph 1·56 days, egg vaccine 1·2 days.

The superiority of A20 was probably due merely to the fact that its virus content was distinctly higher than that of the calf lymph or the first passage egg material B1. Titrations on the chorioallantois gave the following titres at the time when most of the vaccinations were being done: calf lymph 4076, $1 \times 10^7$ (pocks produced per c.cm.); egg vaccine B1, $1{\cdot}6 \times 10^7$; egg vaccine A20, $2{\cdot}8 \times 10^8$. The virus titres of these batches were also determined about four months after their preparation when the vaccinations had been compared. The calf lymph had shown no appreciable fall while the titre of B1 was 1/5–1/10 of the original and that of A20 1/2–1/4 of its original titre.

I am indebted to Dr. F. M. Burnet for the unpublished data from which Fig. 3 is constructed. This shows the rate at which the titres of two egg vaccine preparations (glycerolated) and of calf lymph vaccine diminished when stored in the refrigerator at about 4° C., at room temperature in the dark and at 37° C. All titrations were made by the chorioallantoic method. These results also show that calf lymph retains its titre on storage somewhat better than the egg vaccines. The differences are not great and may be largely accounted for by the fact that the calf lymph had already been stored in the cold for two months before the experiment was started.

*Attempted Application of the Hirst Test to the Titration of Vaccine Virus.*

There would be obvious advantages if material for vaccination could be satisfactorily titrated by a simple *in vitro* test, and a number of experiments directed toward this end have been carried out, partly in collaboration with Dr. Anderson of the Commonwealth Serum Laboratories.

Neither freshly harvested calf lymph nor the glycerolated material gave a satisfactory agglutination of fowl red-cells, although the titre of virus particles as judged by chorioallantoic inoculation was at least as high as in the active egg-vaccine samples. It is presumed that the failure of the reaction is due to the presence of inhibitory substances in the calf lymph, but no adequate demonstration of such substances has been made.

On the other hand vaccinia virus grown on the chorioallantoic membrane showed when freshly prepared an approximately constant relationship between the Hirst titre, and the count of the chorioallantoic membrane. The figures in Table 4 are representative:

TABLE 4.

*Relation between titres by Hirst test and by chorioallantoic titration.*

| | Egg vaccine batch No. (Freshly prepared.) | | | | |
|---|---|---|---|---|---|
| | B1. | A20. | C1. | C2. | C4. |
| Amount of suspension needed to give ± Hirst reaction | 0·015 | 0·004 | 0·008 | 0·008 | 0·008 |
| Titre on chorioallantois | $1{\cdot}0 \times 10^7$ | $2{\cdot}8 \times 10^8$ | $1{\cdot}0 \times 10^7$ | $1{\cdot}8 \times 10^7$ | $2{\cdot}4 \times 10^7$ |

As in the case of influenza-virus preparations (Hirst, 1942) the Hirst titre of an active vaccinia-virus preparation falls more slowly than the titre determined by chorioallantoic titration. Formalin-treated material may retain its full Hirst titre though completely inactive on the chorioallantois. It is therefore impossible to use the Hirst test as a measure of active virus even in egg vaccines. The most that can be said is that if a freshly prepared egg vaccine fails to give a typical Hirst reaction when diluted 1: 100 its virus content is abnormally low and it should not be used for human vaccination.

## DISCUSSION.

The results of this study have established the practicability of Hirst's method for the titration of specific antibody following Jennerian vaccination. The results obtained are on the whole of the type which would have been expected on *a priori* reasoning, the salient features being:

1. The serum of unvaccinated persons contains no antibody demonstrable by the method.

2. Following effective vaccination all previously uninoculated subjects show a moderate to high level of antibody after three weeks.

3. The antibody titre falls rapidly (Nagler, 1942), but about half the previously vaccinated subjects in the group tested showed some residual antibody. There was a significant but no absolute correlation between the presence of antibody and the type of clinical response. Of those showing a primary reaction, 75 p.c. had no detectable antibody, while in the groups giving accelerated and immune reactions the percentages were 45 and 33 respectively.

4. In previously vaccinated individuals the antibody response is determined chiefly by the character of the lesion provoked, the more this approaches the primary type the greater the antibody rise.

From these results certain practical considerations emerge. For primary vaccinations all that is required is to produce a typical lesion as small as practicable and with a minimum of damage to the skin. These requirements are admirably fulfilled by the multiple pressure method. On the other hand if the acute antibody rise is in any sense an indication of the additional immunity conferred, the aim in secondary vaccinations should be to produce as definite a lesion as possible. It is the general experience that in secondary vaccinations the proportion of active "takes" can be increased by the use of more drastic methods for inserting the virus. Where the maximum immunity is desired it would seem logical to vaccinate first by the standard multiple pressure method and ten days later revaccinate all those showing either immune reactions or failures to take (i.e. all without a definite crust or vesicle) by scarification and two insertions.

The problem of whether egg-grown vaccinia virus is a satisfactory vaccinating agent can be answered finally by experience in a smallpox epidemic. As far as can be judged by the appearance of the lesions in persons of different immunity levels and by the antibody response, there is no detectable difference between calf lymph and egg vaccine from one to twenty passages removed from the calf lymph. In both types the effectiveness of the vaccine appears to be determined only by the number of active particles present in the material when it is used. In favour of calf lymph is its slightly better keeping qualities while the special advantage of the egg vaccine is its freedom *ab initio* from bacteria and the rapidity with which large amounts could be prepared in an emergency.

## SUMMARY.

Vaccinia virus grown on the chorioallantois of developing chick embryos has been used for the vaccination of more than 1,500 subjects with clinically satisfactory results.

With an adequately high titre preparation the proportion of failures to take is very low (0·24 p.c.) but with virus of lower titre the proportion of successes may be inferior to those obtained with calf lymph.

The antibody response as judged by red cell agglutination methods (Hirst's test) is of the same order in subjects vaccinated with egg vaccines or calf lymph.

The antibody response in revaccinated persons is correlated with the clinical type of their reaction, being greatest in those who give a primary type reaction, least in those showing an immune reaction.

More than half of a group of persons who had been vaccinated at various times previously showed some residual antibody.

Glycerolated egg vaccine loses titre significantly more rapidly than matured calf lymph.

Fowl red-cell agglutination is not a suitable method for titrating the activity of vaccinia virus; calf lymph of high virus content gives only equivocal agglutination and egg vaccine may show a sharp drop in its content of active virus with little change in its agglutinating power.

*Acknowledgments.* I wish to acknowledge the kind co-operation of Drs. M. Blanch, D. Officer and S. Nash, who carried out the vaccination of children in the kindergartens, of Dr. Dale and the Health Officers of the City of Melbourne who volunteered for the vaccination and Dr. F. M. Burnet for constant helpful advice and interest in the work. I am also much indebted to Miss Ellen Clark for her help in vaccinating and bleeding many of the subjects.

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# THE METABOLISM OF STARVING LEAVES

## 4. RESPIRATION RATE AND METABOLISM OF LEAVES OF KIKUYU GRASS DURING AIR-NITROGEN TRANSFERS

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*(Accepted for publication, 21st December, 1943.)*

In a previous paper (Wood, Cruickshank and Kuchel, 1943) data were presented which suggested that when leaves were placed in an atmosphere of nitrogen then the rate of protein hydrolysis was less than that which occurred in air; and further, that under these conditions amino acids accumulated but amides and ammonia were not formed. The experiments there described were preliminary ones and based upon relatively infrequent samples. In the present paper we explore more fully the changes in respiration rate, and in amounts of various carbohydrates, nitrogen compounds and organic acids, which occur when leaves are transferred from air to nitrogen and *vice versa* at various stages of starvation. The changes are described in leaves of Kikuyu grass (*Pennisetum clandestinum*, Hockst.), a plant in which very little glutamine but much asparagine occurs during starvation.

### EXPERIMENTAL.

Plants were grown in plots in the field, transferred to the laboratory and the leaves severed at the ligule; the leaves were bulked, thoroughly mixed and sampled, and aliquots placed in black containers in water baths at $24{\cdot}50° \pm 0{\cdot}02°$ C. Air or nitrogen streams were passed over the samples at a rate of 4 litres per hour. 20 gm. samples were used for determination of respiration rate and 40 gm. samples for analytical material. Samples were withdrawn for analysis usually at intervals of 24 hours. The method for determination of respiration rate and analytical methods employed were similar to those already described by Wood, Cruickshank and Kuchel (1943). Respiration rate is reported as mg. $CO_2$ per 20 gm. original fresh weight per hour. All other compounds are reported as mg. per 20 gm. original fresh weight. The term Total-N in the figures is used for the sum of the nitrogen fractions actually estimated.

*Experiment IX.* The leaf samples were divided into three series. In Series A a stream of air was passed continuously over the leaves for 144 hrs. In Series B, pure nitrogen was passed for the first 24 hrs. when it was replaced by air and the air stream continued up to 144 hrs. In Series C, nitrogen was passed for the first 48 hrs. when it was replaced by air and the air stream continued up to 144 hrs. Respiration rates and amounts of various metabolites for the three series are compared and figured in Fig. 1.

*Experiment X.* The leaf samples were divided into four series. In Series D, air was passed continuously over the leaves for 168 hrs. In Series E air was passed up to 24 hrs. when the air stream was replaced by nitrogen which was continued up to 96 hrs., the nitrogen was then replaced by air which continued up to 168 hrs. In Series F air was passed up to 48 hrs. when it was replaced by nitrogen which continued up to 120 hrs., the nitrogen was then replaced by air which continued up to 192 hrs. In Series G air was passed up to 96 hrs. when it was replaced by nitrogen which continued up to 168 hrs., the nitrogen was then replaced by air up to 192 hrs. Data for Series D, E, F and G are shown graphically in Figs. 2, 3, 4 and 5 respectively.

### DISCUSSION.

#### *Changes during Air-Nitrogen transfers and* vice versa.

Examination of the data of the two experiments (Figs. 1–5) permits the following general statements to be made.

Respiration curves in air exhibit a characteristic climacteric "hump";

transference to nitrogen from air causes the respiration rate to fall to a low value and no climacteric occurs in nitrogen. On replacing the nitrogen again with air the respiration rate rises to a high value many times (up to 21 times, Fig 1, series C) that of the previous value in nitrogen; in this subsequent period in air the respiration rate exhibits a climacteric hump similar to that shown by leaves which have been kept continuously in air. It appears from the respiration curves therefore that sojourn in nitrogen has not affected the operation of systems within the leaves but has delayed the appearance of reactions associated with the appearance of the climacteric.

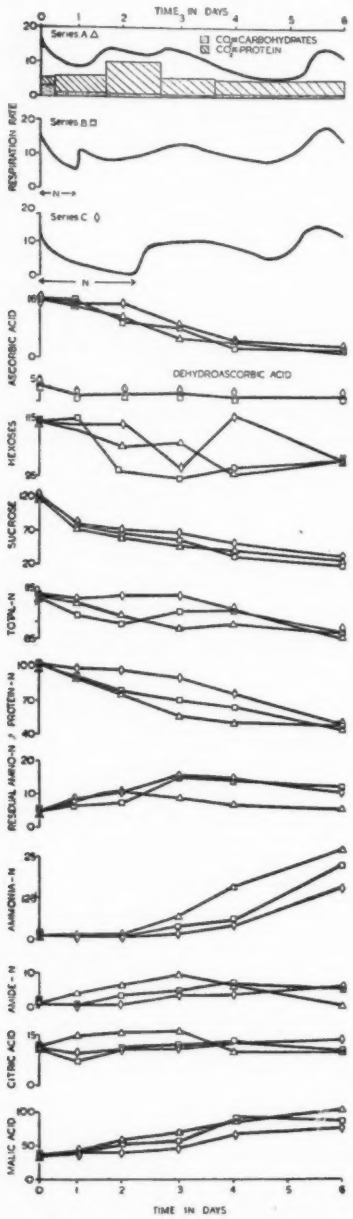


**Fig. 1.**

The amount of hexose sugars falls slightly during the starvation period but does not show significant differences between treatments; on the other hand, the amount of sucrose falls markedly during early stages of starvation and at the end of the experiments has practically all disappeared. In both air and nitrogen sucrose is utilized at approximately the same rate so that it is clear that the climacteric hump in air is not associated with increased utilization of soluble carbohydrates.

It should be noted that during the first hours of starvation the rate of $CO_2$– production in nitrogen is approximately the same as that in air (Fig. 1). Since, as will appear later in this paper, during early stages of starvation $CO_2$ is derived largely, though not entirely, from carbohydrates, it is probable that the Pasteur effect observed in yeasts and in predominantly starchy plant storage organs may also be operative in leaves during these early stages of starvation.

Sojourn in nitrogen inhibits oxidation of ascorbic acid but on transference to air the amount of ascorbic acid falls steadily with time. The amount of dehydroascorbic acid is relatively small and there is no evidence that nitrogen affects the rate of loss of dehydroascorbic acid.

Protein hydrolysis continues in nitrogen but the rate of protein loss is less in nitrogen than in air. In nitrogen amino acids only accumulate but there is no accumulation of amides or of ammonia; on transference to air from nitrogen residual amino acids decrease in amount whilst amides and ammonia increase in amount; in late stages of starvation amides decrease in amount

whilst ammonia accumulates. It should be noted that even in cases where the amino acid content has already reached a maximum and is decreasing in amount in air, then transference to nitrogen suppresses formation of amides and of ammonia but amino acids again accumulate slowly.

Malic and citric acids do not accumulate in nitrogen but increase in amount when leaves are transferred to air.

*The Form of the Respiration Curves in Air.*

It has been shown above that carbohydrates decrease in amount at approximately the same rate in nitrogen and in air and also that when leaves are transferred from nitrogen to air they may enter upon the climacteric phase and the rate of $CO_2$-production is many times that of the previous value in nitrogen. It is clear that substances other than carbohydrates are oxidized and ultimately produce $CO_2$ during this phase. Yemm (1935), Vickery *et al.* (1933) and Wood, Cruickshank and Kuchel (1943) have shown previously that the loss of carbohydrates is insufficient to account for the observed $CO_2$-production, and surmised that nitrogen compounds were oxidized during this stage.

In the present experiments, as in those described previously by Wood, Cruickshank and Kuchel (1943), the climacteric hump in air occurs between the maximum residual amino-acid content and the maximum asparagine content; the later decrease in respiration rate is associated with decreasing amounts of asparagine and increasing amounts of ammonia. It is probable therefore that amino acids derived from protein are in part transformed into asparagine and in part oxidized to $CO_2$.

It should be possible therefore to calculate approximately from the data the amounts of $CO_2$ derived from carbohydrate sources and from nitrogen sources. Leaves of Kikuyu grass are excellent for this purpose. Unlike some other grass leaves, whose metabolism will be reported later in this series, the sum of the protein-N, residual amino-N, amide-N and ammonia-N remains approximately constant over the whole starvation period as may be seen from the individual experiments in Figs. 1–5. Approximately all the nitrogen in Kikuyu leaves, therefore, is concerned in the transformations described above. The amounts of $CO_2$ derived from different sources have been calculated as follows: (i) from the carbohydrate figures on successive days, assuming that all the carbohydrate lost has been oxidized to $CO_2$; (ii) from the changes in amounts on successive days of protein-N, residual amino N and asparagine-N. In order to do this it is necessary to obtain the carbon-equivalent of the nitrogen in these compounds. Since proteins in general contain about 17 p.c. N and 52 p.c. C we have taken $3 \times$ Protein-N as a measure of protein-carbon; similarly for residual amino acids derived from the protein, $3 \times$ Residual Amino-N is taken as a measure of amino-acid-carbon. $3{\cdot}43 \times$ Amide-N gives a measure of asparagine carbon. The algebraic sum of the differences in amounts of carbon of protein, amino acids and asparagine between any two periods, gives a measure of the carbon not accounted for by amide-formation. It is assumed that this carbon is all oxidized to $CO_2$.

The calculated amounts of $CO_2$ accounted for by carbohydrate loss and transformations and oxidations of nitrogen compounds, and calculated as a mean hourly rate over the 24-hr. period, are given for the two series of leaves kept continuously in air in Experiments IX and X (Figs. 1 and 2). It will be seen that during the first 24 hrs. of starvation carbohydrates contribute largely to $CO_2$–production: the total amount of $CO_2$ produced by respiration is not wholly accounted for, but the carbohydrates estimated included only hexoses and sucrose; other insoluble carbohydrates doubtless also contribute to the respiratory substrate during this period. Thereafter nitrogen compounds contribute to the formation of an increasing and relatively large amount of $CO_2$ produced; and in late stages of starvation practically all the $CO_2$ produced is accounted for in terms of oxidation of nitrogen compounds.

*Mechanism of Transformations and Oxidation.*

Although inter-relationships among nitrogen compounds, carbohydrates, organic acids and respiration rate are obvious from the data, the mechanism of such inter-relations remains speculative. In a previous paper (Wood, Cruickshank and Kuchel, 1943) it was suggested that in leaves the metabolic scheme included oxidation of carbohydrates through a Krebs' cycle of organic acid mediators some of which (e.g. oxalacetic and $\alpha$-ketoglutaric acids) formed the carbon source of the amides asparagine and glutamine; oxidation of amino acids by amino-oxidases; formation of glutamic acid by glutamic enzyme and of aspartic acid by aminophorases. Such a scheme is similar in its essentials to that proposed by Chibnall (1939) and Vickery (1939). All stages in this scheme are controlled by different enzymes or enzyme systems.

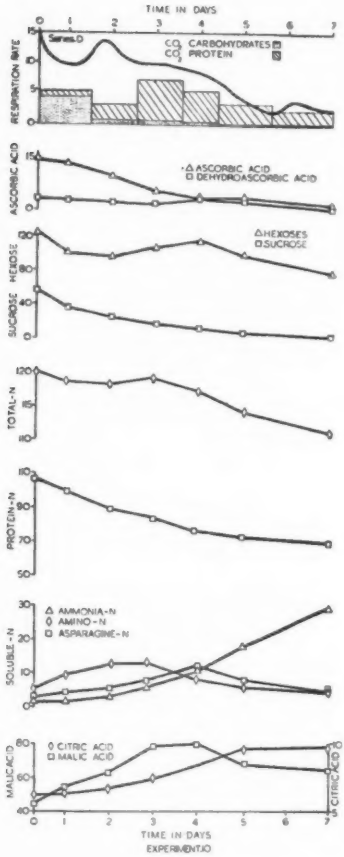


**Fig. 2.**

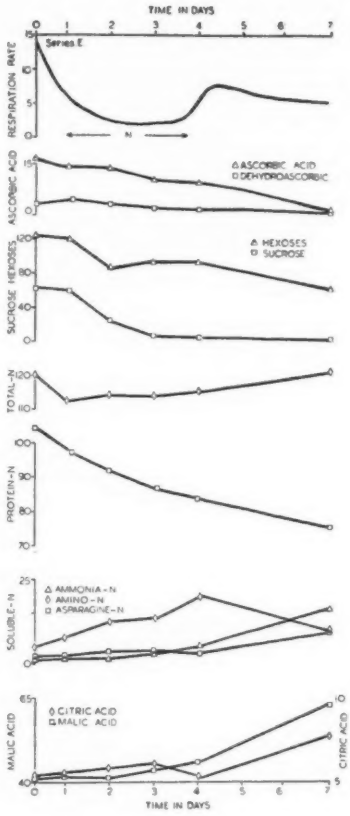


**Fig. 3.**

In these figures amino-N refers to Residual amino-N.

In all leaves so far investigated (Vickery *et al.*, 1937, 1939; Yemm, 1937; Mothes, 1926; Wood *et al.*, 1943) it is apparent that a common sequence in all leaves consists in hydrolysis of proteins to amino acids, oxidation of amino acids with subsequent amide formation and production of ammonia. There are, however, marked differences in amounts of the two amides in different leaves. Kikuyu grass resembles tobacco (Vickery *et al.*, 1937) in that relatively large amounts of asparagine are formed during starvation, but very little glutamine. Barley leaves (Yemm, 1937) produce relatively large amounts of both glutamine and asparagine, and oat leaves investigated by us (unpublished) are similar in this respect to barley. Rhubarb leaves (Vickery *et al.*, 1939) produce no asparagine but only glutamine. Such differences are explicable if different leaves contain varying amounts or are lacking in the glutamic or aminophorase enzymes presumed responsible for amide formation.

But the differences between leaves of different species of plants are reflected not only in the amount and nature of the amides but also in the amounts of malic and citric acids and their behaviour during starvation. Leaves of Kikuyu grass

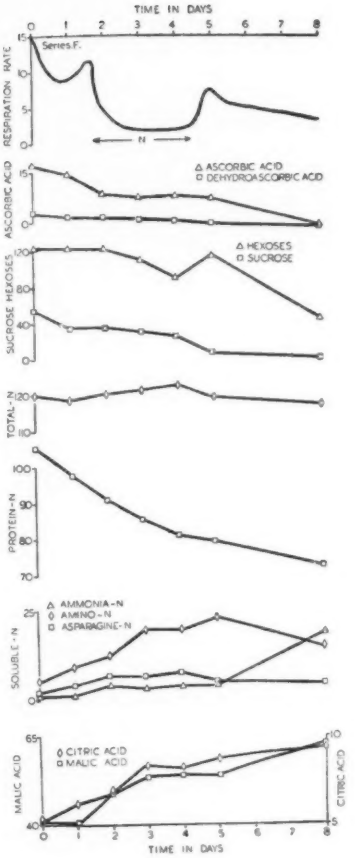


**Fig. 4.**

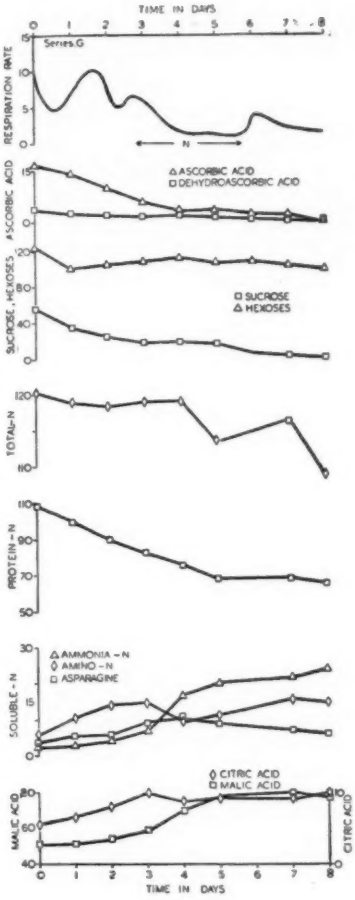


**Fig. 5.**

In these figures amino-N refers to Residual amino-N.

differ from those of the two other plants which have been thoroughly investigated (*viz.* tobacco and rhubarb, by Vickery and his collaborators) in the marked accumulation of malic acid which occurs during starvation.

These three cases may be summarized as follows:

*Kikuyu.* Total acids increase in amount during starvation. Malic acid increases up to a late stage of starvation when it decreases in amount. Citric acid increases to a maximum which is earlier than that of malic acid, and later decreases in amount.

*Tobacco.* Total acids remain approximately constant in amount during starvation. Malic acid decreases markedly in amount but an equivalent amount of citric acid appears in the leaves.

*Rhubarb.* Total acids decrease during starvation. Both malic and citric acids decrease in amount; it is probable in this leaf that some of the acids are respired to $CO_2$.

In the experiments described in the present paper, as well as in those of Wood, Cruickshank and Kuchel (1943), increase in amount of citric acid is associated with initial loss of carbohydrate, whilst increase in amount of malic acid is associated with (i) increase in amount of asparagine during early stages of starvation and (ii) decrease in amount of asparagine and increase in amount of ammonia during later stages. It is especially apparent in Experiment X that malic acid increases contemporaneously with asparagine when air is admitted to the leaves after a relatively long sojourn in nitrogen, and at a time when carbohydrates are almost exhausted.

During the course of starvation the pH of the extracts prepared from the dried leaf material, before precipitation of protein, decreases progressively. It is probable that much of this increase in acidity is due to malic acid and its salts. We have calculated from the ammonia data of each day the equivalent of malic acid if all the ammonia were present as ammonium malate. At all stages of starvation, except sometimes on the last day there is sufficient malic acid present to neutralize all the ammonia formed. During early stages of starvation the amount of malic acid present is greater than that required to neutralize all ammonia present; later, ammonia and malic acid are present in approximately equivalent amounts. It appears therefore that at this stage the malic acid is probably present as the diammonium salt.

It would appear from the foregoing that most of the malic acid in Kikuyu is derived from nitrogeneous sources. However, this cannot be the only source of malic acid. Vickery's data (1939) for rhubarb show that although malic and citric acids decrease in amount in darkness, yet they increase in amount in the light or when cultured in the dark on glucose solutions. Origin from a carbohydrate source at least in part is, therefore, also probable. Such an origin in part is also implicit if the Krebs' cycle is admitted as a probable mechanism during synthesis of nitrogen compounds, for in this cycle the acids behave simply as "mediators" whereby carbohydrate is oxidized. If all carbohydrate was completely oxidized then there would be no change in amounts of organic acids unless from another source. Vickery's data (1937) showing the equivalent production of citric acid from malic acid in tobacco is supporting evidence for the operation of a cycle of organic acids.

The apparently conflicting data described for Kikuyu, rhubarb and tobacco are reconciled if the operation of a cycle of organic acids, such as that of Krebs, is postulated; the observed differences in amounts of different organic acids are then attributable to different amounts or activities of enzyme systems, e.g. it is possible that the oxidation of malic acid to citric acid proceeds more rapidly in tobacco than in Kikuyu. Also loss of malic acid, apparent in later stages of starvation in all experiments is apparently more rapid in rhubarb than in the other leaves investigated. It is also probable, as discussed by Vickery *et al.* (1939) that the carbohydrate status of the plant and the relative efficiencies of glutamine and asparagine formation may differ in different species or even in the same species at different times. Substantiation of these postulates must await further work on enzyme systems, but the hypothesis outlined earlier accounts for all the phenomena concerned with nitrogen, carbohydrate and organic acid metabolism which have been observed in starving leaves.

## SUMMARY.

The metabolism of leaves of Kikuyu grass has been investigated when the leaves are transferred from nitrogen to air and *vice versa* at various stages of starvation. Respiration rate and amounts of hexoses, sucrose, protein, amino acids, amides, ammonia, malic and citric acids have been determined at 24-hour intervals.

It has been shown that during early stages of starvation, when carbohydrate substrates chiefly are being utilized, rate of $CO_2$-production is approximately the same in nitrogen and in air. During later stages of starvation the respiration rate is many times greater in air than in nitrogen. In nitrogen the respiration rate/time curve does not exhibit a climacteric rise. It has been shown that in air the climacteric rise is associated with utilization of nitrogen compounds.

In nitrogen, ascorbic acid does not decrease in amount, as in air. The rate of protein loss in nitrogen is considerably less than in air, and in nitrogen amino acids only accumulate but amides and ammonia are not formed as they are in

air. In nitrogen, malic and citric acids do not increase in amount, but increase when leaves are transferred to air.

Amide and organic acid metabolism in this grass is compared with that in other leaves and the probable mechanism of the metabolic processes is discussed.

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# THE INFLUENCE OF METAL IONS ON GLUCOSE FORMATION BY THE LIVER

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*(Accepted for publication 22nd December, 1943.)*

Studies on the formation of glucose from glycogen by liver extracts have shown that there are two controlling factors, namely, the concentration of inorganic phosphate (Ostern, Herbert and Holmes, 1939) and the pH (Fantl *et al.*, 1942a). The results of subsequent work relating to the influence of certain metal ions on polysaccharide breakdown in liver extracts are presented here.

## EXPERIMENTAL.

To exclude the possibility of reducing substances being formed by diastase, the experiments were carried out with extracts obtained from rabbit livers perfused *in situ* with saline. Glucose was generally estimated as reducing substances by the Shaffer-Somogyi technique as modified by Harding and Downs (1933) since, as shown in Table 1 (Expts. 1, 2, 4, 5), the results obtained when these values were checked by fermentation (Somogyi, 1938), and glycogen estimations (Good *et al.*, 1933) agreed closely. In cases where cysteine was added more reliance was placed on the glycogen estimations.

The phosphate concentration was usually M/45 for reasons stated previously (Fantl *et al.*, 1942a) and the results shown in Table 1 illustrate that in the presence of magnesium ions very little, and in the case of addition of manganese ions no, accumulation of hexose phosphates occurred.

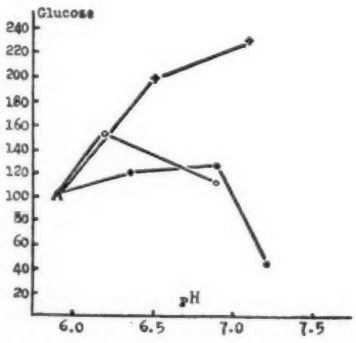



**Fig. 1.** 1: 6 diluted liver extracts containing M/45 Sörensen's phosphate buffer, $10^{-3}$ M. $Na_2SO_4$ and 1 p.c. starch or glycogen incubated for 1 hour at 37° C. The values at pH 5·9 (A) were taken as 100. Each sign represents an independent experiment.

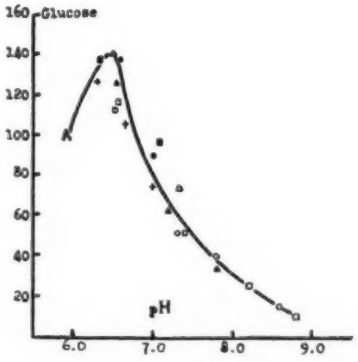



**Fig. 2.** 1: 6 diluted liver extracts containing $10^{-6}$ M. $MgSO_4$ incubated for 1 hour at 37° C. Values at pH 5·9 (A) taken as 100. Each sign indicates an independent experiment.

[1] Working under a full-time grant from the National Health and Medical Research Council.

*The Influence of Magnesium Ions.*

Extracts of rabbit livers have a pH activity curve for polysaccharide breakdown varying over a considerable range (Fig. 1). However, much more uniform results are obtained when $10^{-3}$ M. magnesium sulphate is added (Fig.2).

The curve obtained is similar to previously published results, namely that the enzyme system has an optimum pH at $6{\cdot}5 \pm 0{\cdot}1$ (Fantl *et al.*, 1942a) and represents the activity curve for liver phosphorylase. Since the magnesium concentration used is approximately the same as that found in normal livers (Eichelberger and McLean, 1942), it seems likely that the curve with an optimum at pH 6·5 reproduces events as they occur *in vivo*.

TABLE 1.

*1:6 diluted liver extracts containing M/45 Sörensen's phosphate buffer and $10^{-3}$ M. $MgSO_4$, $MnSO_4$ or $ZnSO_4$ (unless otherwise specified) were incubated for 2 hours at 37° C. with either 1 p.c. starch or glycogen.*

| Expt. No. | Conditions. | pH. | Mg. glucose formed per 100 ml. reaction mixture estimated by: Reduction. | Fermentation. | Glycogen loss. | Phosphorus uptake (P. × 5·8). | Oxygen consumption in emm. |
|---|---|---|---|---|---|---|---|
| 1 | Glycogen + Mg. | 6·4 | 410 | 390 | 390 | 0 | |
| 2 | Starch + Mg. | 6·2 | 550 | 530 | — | 0 | |
| 3 | Starch + Mg. | 6·6 | 490 | | | | |
| | Starch + Mn. | 6·6 | 380 | | | | |
| | Starch + Mg. + Mn. | 6·6 | 380 | | | | |
| 4 | Starch + Mg. | 6·4 | 485 | 470 | | 20 | 70 |
| | Starch + Mg. + 250 mg. p.c. cysteine HCl | 6·4 | 460 | 450 | | 0 | 160 |
| | Starch + Mn. | 6·4 | 360 | 360 | | 0 | 95 |
| | Starch + Mn. + 250 mg. p.c. cysteine HCl | 6·4 | 340 | 340 | | 0 | 200 |
| 5 | Glycogen + Mg. | 6·4 | 520 | | 590 | 60 | |
| | Glycogen + Mg. + 300 mg. p.c. cysteine HCl | 6·3 | — | | 640 | 0 | |
| | Glycogen + Mn. | 6·4 | 295 | | 295 | 0 | |
| | Glycogen + Mn. + 300 mg. p.c. cysteine HCl | 6·3 | 290 | | 280 | 0 | |
| | Glycogen + Zn. | 6·3 | 130 | | 135 | 20 | |
| | Glycogen + Zn. + 300 mg. p.c. cysteine HCl | 6·3 | | | 180 | 30 | |
| 6 | Starch + Mg. | 6·4 | 410 | | | 0 | |
| | Starch + Mg. + 250 mg. p.c. cysteine HCl | 6·4 | 440 | | | 5 | |
| | Starch + Zn. | 6·4 | 210 | | | 110 | |
| | Starch + Zn. + 250 mg. p.c. cysteine HCl | 6·4 | 440 | | | 5 | |
| 7 | Starch + Mg. | 6·4 | 415 | | | 45 | |
| | Starch + Mg. + glycine | 6·4 | 470 | | | 65 | |
| | Starch + Zn. | 6·4 | 160 | | | 45 | |
| | Starch + Zn. + glycine | 6·4 | 190 | | | 25 | |
| 8 | Glycogen + $10^{-3}$ M. $Na_2SO_4$ | 6·6 | 545 | | | | |
| | Glycogen + $10^{-4}$ M. $ZnSO_4$ | 6·6 | 480 | | | | |
| | Glycogen + $10^{-3}$ M. $ZnSO_4$ | 6·6 | 295 | | | | |

*The Influence of Manganese Ions.*

The element manganese shows activating properties similar to those of magnesium for several enzymes involved in carbohydrate breakdown, for example: phosphoglucomutase (Cori *et al.*, 1938) and the yeast carboxylase concerned in the breakdown of pyruvic acid (Lohmann and Kossel, 1939). However, when manganese sulphate, in equimolecular concentrations to magnesium, was added to liver extracts the glucose production from polysaccharides was inhibited between pH 6·2 and 7·0 (Fig. 3.).

At the optimal pH the degree of inhibition was from 25–45 p.c. in 9 out of 10 experiments using liver extracts, and in 2 out of 3 experiments with liver pulp. The inhibition by manganese could not be abolished by the addition of equimolecular concentrations of magnesium (Table 1, Expt. 3).

No accumulation of hexose phosphates was detected in any of the liver extracts containing manganese, and it is therefore probable that manganese ions exert this effect on the phosphorylase.

Cori and Cori (1940) have observed that muscle phosphorylase is stablized by — SH compounds, and it is suggested that these substances react with metal ions inhibitory to the enzyme. However, the addition of cysteine to liver extracts containing manganese did not significantly alter the inhibitory effect (see Table 1, Expts. 4 and 5).

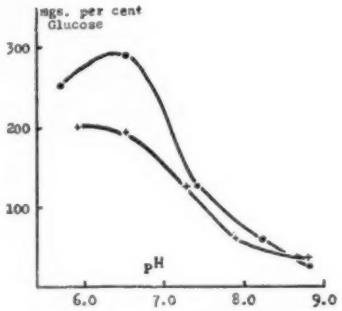



**Fig. 3.** Liver extracts incubated for 1 hour with 1 p.c. starch. '●' represents values obtained when the reaction mixture contained $10^{-3}$ M. $MgSO_4$; '+' $10^{-3}$ $MnSO_4$. Values shown represent mg. glucose formed per 100 ml. reaction mixture.

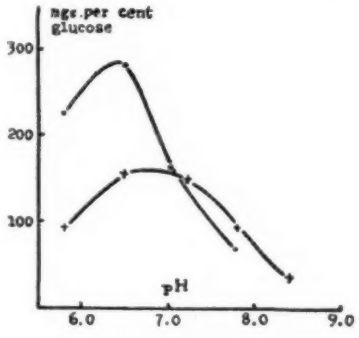



**Fig. 4.** Extracts as before. Experiments indicated by '●' contained $10^{-3}$ M. $MgSO_4$. Those indicated by '+' contained $10^{-3}$ M. $ZnSO_4$. Values shown represent mg. glucose formed per 100 ml. reaction mixture.

It does not seem likely that manganese catalyzes an oxidation of the enzyme for, although the oxygen consumption of manganese-containing extracts is higher than in the control experiments and is further increased by the addition of cysteine, no difference in the glucose production parallel to the higher oxygen consumption could be observed (see Table 1, Expt. 4).

*The Influence of Zinc Ions.*

The conversion of glucose–1– to glucose–6–phosphate in muscle extracts (Cori *et al.*, 1938; Gill and Lehmann, 1939; Fantl and Rome, 1942b; Lehmann, 1939) and the breakdown of pyruvic acid in yeast (Lohmann and Kossel, 1939) are both inhibited by zinc ions, although the initial step of glycogen breakdown to glucose–1–phosphate in muscle does not seem to be influenced by this metal (Lehmann, 1939). However, when $10^{-3}$ M. zinc ions were added to liver extracts there was a marked reduction in the glucose formation in the range pH 5·8–7, although above neutral point zinc had no influence (Fig. 4).

The degree of inhibition was dependent upon the concentration of zinc, $10^{-4}$ M. being sufficient to produce some inactivation (Table 1, Expt. 8). The inhibition in 19 experiments carried out with liver extracts containing $10^{-3}$ M. zinc at pH 6·5±0·1 was between 40 to 70 p.c.

The adverse influence of zinc ions on glucose production was also observed in liver pulp at the optimal pH, but the inhibition was smaller, varying between 15 and 40 p.c. The inhibition by zinc was not abolished by the addition of equimolecular concentration of magnesium as shown in Table 2.

TABLE 2.

| pH. | + $10^{-3}$ M. $MgSO_4$. | + $10^{-3}$ M. $ZnSO_4$. | + $10^{-3}$ M. $MgSO_4$ + $10^{-3}$ M. $ZnSO_4$. |
|---|---|---|---|
| 5·8 | 230 | 100 | 120 |
| 6·5 | 280 | 160 | 170 |
| 7·1 | 160 | 150 | 150 |
| 7·8 | 70 | 90 | 100 |
| 8·4 | — | 30 | 40 |

Experimental conditions similar to those given in Table 1 (incubation time was 1 hour, substrate 1 p.c. glycogen).

Since the experiments were carried out in a medium containing inorganic phosphate, precipitation of zinc phosphates occurs and it was thought that the decrease of available inorganic phosphate for glycogen breakdown may be responsible for this inhibition. However, extracts containing M/60 and M/45 phosphate buffer at pH 6·6 formed glucose from glycogen at practically the same rate.

The inhibitory influence of zinc may be due to one of several factors. Firstly, as quoted above, zinc inhibits the conversion of glucose–1–phosphate to its isomer, glucose–6–phosphate, and according to Cori *et al.* (1938) an accumulation of the former inhibits phosphorylase activity. Secondly, zinc may have a direct influence on liver phosphorylase or it is possible that the precipitation of zinc phosphates may adsorb this enzyme, thus inactivating it.

Lehmann and Pollak (1942) observed that calcium or magnesium phosphates can be kept in solution by the addition of amino acids, cysteine being more effective than glycine. Applying this principle, it was found that zinc ions can be kept in solution by both glycine and cysteine. 2 mols. of either glycine or cysteine, in the absence of inorganic phosphate, can dissolve a maximum amount of 1 gram atom of zinc ion. Eisenbrand and Wegel (1941) claim to have obtained evidence for a compound with the ratio of zinc: nitrogen = 1: 1. However, even with an excess of zinc hydroxide and a concentrated glycine solution, we were able to isolate only the known compound: $Zn(O.CO.CH_2NH_2)_2 H_2O$. (Calculated: 28·38 p.c. zinc, 12·1 p.c. nitrogen. Found: 28·4 p.c. zinc, 12·2 p.c. nitrogen.) Zinc glycinate is not stable in the presence of inoganic phosphate, an equilibrium between zinc-phosphate and zinc-glycinate being established, and even in the alkaline range more than 20 mols. of glycine are required to keep 1 gram atom of zinc in solution (Fig. 5, Curve B). Cysteine at acid pH forms an insoluble mercaptide with zinc salts which dissolves on addition of alkali. Zinc cysteinate is stable in the presence of inorganic phosphate above pH 6·3 (Fig. 5, Curve A) and, consequently, only 2 mols. of cysteine are required for each gram atom of zinc.

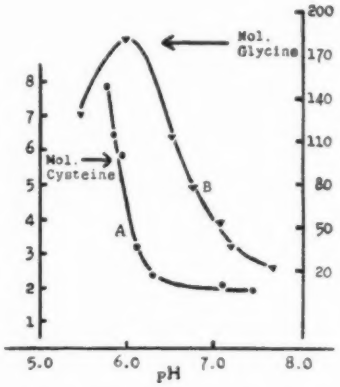



**Fig. 5.** Curve A. The results obtained when a $10^{-2}$ M. $ZnSO_4$ was added to 2 ml. M/45 phosphate buffer and 1 ml. of M/15 cysteine, until the Tyndall phenomenon was observed. The pHs were then measured with the glass electrode.

Curve B. The results of a similar experiment with glycine.

Although the addition of 0·25 M. glycine is sufficient to keep the zinc ions in solution in a phosphate-containing medium, it does not lessen the inhibitory effect of zinc on glucose formation (Table 1, Expt. 7).

The addition of cysteine to zinc-containing liver extracts gave varying results as shown in Table 1, Expts. 5 and 6. In some cases cysteine abolished the inhibitory influence of zinc on polysaccharide breakdown, although in others only slight, or no, beneficial effect could be seen. The fact that in some experiments the phosphate uptake was the same in both the magnesium and the zinc series, and the inability of magnesium to reverse the zinc effect, suggest that zinc exerts its inhibitory influence directly on the phosphorylase.

## DISCUSSION.

Since it is established that metals, such as magnesium, manganese and zinc, occur in tissues and in the purest preparations of enzymes, it is of interest to know how they influence the various enzymatic reactions.

Magnesium ions are apparently not essential for the enzymatic breakdown of glycogen to glucose, although they frequently accelerate the reactions involved therein. For example, the conversion glucose–1– to glucose–6–phosphate is accelerated by the addition of magnesium, and Cori *et al.* (1938) have assumed that the effect of magnesium ions on purified muscle phosphorylase is due to removal of the inhibiting glucose–1–phosphate. This explanation cannot be applied to liver extracts, however, since the formed glucose–1–phosphate is continually removed by the action of phosphoglucomutase, and the glucose–6–phosphate broken down into its components, as shown by the fact that the concentration of inorganic

phosphate remains unchanged at the end of the incubation period (Table 1). This indicates that magnesium exerts a regulating influence on liver phosphorylase itself. At any rate, the magnesium concentration of a normal liver is approximately the same as in the experiments reported, and the conclusion, that the curve obtained with organ extracts reflects events as they occur in the cell, seems therefore to be justified.

The behaviour of manganese is, however, rather unexpected since it is frequently considered to have similar acceleratory properties to magnesium in the reactions under discussion, and in some cases has been shown to be even more active. It is interesting to note that, whilst manganese inhibits phosphorylase, the other enzymes in liver essential for glucose formation are fully active since in none of the experiments could a change in the concentration of inorganic phosphate be detected. However, although the inhibition by manganese ions is of importance from a theoretical point of view, it is doubtful whether manganese would influence the blood sugar production *in vivo*, since the manganese content of the normal liver has been shown to be of the order of 1·2 to 3·5 mg. per kg. of wet tissue, corresponding to 3 to 6 $\times$ $10^{-5}$ M. manganese (Von Oettingen, 1935).

In all experiments, zinc caused an appreciable inhibition of polysaccharide breakdown, whether the phosphorus uptake was the same (Table 1, Expt. 7) or exceeded the amount taken up in the presence of magnesium ions (Table 1, Expt. 6). In the former case, it appears that the phosphoglucomutase was still active in spite of the presence of zinc and it must, therefore, be concluded that zinc exerted a direct influence on the phosphorylase. In cases where glucose–1–phosphate accumulated, it is obvious that the phosphoglucomutase was primarily inhibited. However, it was possible to activate phosphoglucomutase by the addition of cysteine in agreement with the observations of Lehmann (1939) on the muscle enzyme and still the glucose production in some experiments did not reach the level of the magnesium series (Table 1, Expt. 5). Furthermore, the addition of magnesium ions did not abolish the adverse effect of zinc (Table 2).

Hence it appears that the influence of zinc ions on glycogen breakdown depends upon the activities of phosphorylase and phosphoglucomutase in liver extracts. In the case of a very active phosphorylase and inhibited phosphoglucomutase, $10^{-3}$ M. zinc reduces the breakdown by accumulation of glucose–1–phosphate, whereas, in cases of relatively low phosphorylase activity, it is reasonable to assume that $10^{-3}$ M. zinc inhibits liver phosphorylase directly.

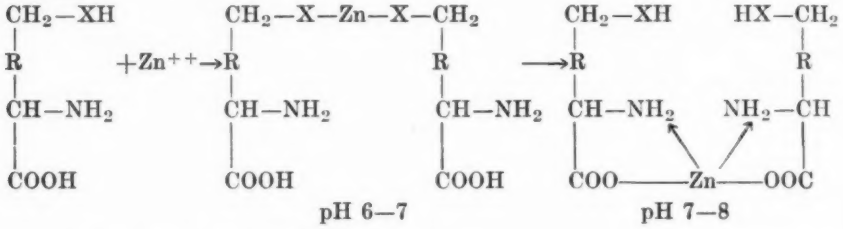
It may be mentioned here that experiments on aqueous extracts of rabbit muscle were carried out under similar conditions. These experiments showed that the glycogen breakdown in muscle extracts, using $10^{-3}$ M. magnesium, manganese or zinc ions, showed no difference in glycogen disappearance and it appears, therefore, that liver phosphorylase is more susceptible to attack by manganese and zinc ions than the muscle enzyme.

According to Scott and Fisher (1935a), the zinc content of fresh pancreas is 20 mg. per kg. and it is considered by these authors (1935b) as a factor in the liberation of insulin and its activity in the body. Further, Eggleton (1940) finds that the zinc content of fresh liver is approximately 5 $\times$ $10^{-4}$ M.—a concentration which already has some influence on the production of glucose *in vitro*.

All experiments indicate that the inhibitory effects of zinc and manganese ions are restricted to the range below pH 7. It is interesting to compare this phenomenon with the behaviour of these ions towards the amino-acids, glycine and cysteine. Near the isoelectric point their ability to keep zinc ions in solution is small and increases with increased hydroxyl ion concentration.

The solubility of manganese in glycine–phosphate solution is smaller than that of zinc and the behaviour of the manganese salts towards cysteine is more complex, since, under aerobic conditions, oxidation occurs.

The experiments with simple amino-acids suggest an explanation of the behaviour of zinc and manganese towards the more complex enzyme molecule itself. In the acid range the metal may react with an essential group of the phosphorylase molecule, thus diminishing its activity, whereas, above pH 7 an intramolecular shift takes place, freeing the essential group. This assumption may be expressed as follows:




where R = the rest of the enzyme molecule
XH=an essential group, possibly an –SH group.

## SUMMARY.

Magnesium ions, in a concentration similar to that found in the normal liver, exert a regulatory effect on phosphorylase in extracts of rabbits' livers. In contrast to the effect with muscle phosphorylase, manganese or zinc ions cause an inhibition of polysaccharide breakdown in liver between pH 6 to 7. With neither metal is the inhibition abolished by the addition of magnesium or glycine, but whereas manganese causes an irreversible inactivation of liver phosphorylase, the inhibitory effect of zinc may be overcome by cysteine.

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# THE OCCURRENCE OF SALMONELLA TYPES IN AUSTRALIA. 1

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There is little information on the occurrence and distribution of Salmonellas in Australia. Stewart, working in the veterinary field, has been the main contributor. He recovered *S. typhi-murium* on several occasions from sheep (1940a), pigs and horses (1940b) and ducks (1939); *S. bovis-morbificans* from sheep and a pig (1940 a and b); *S. cholerae-suis* var. *kunzendorf* from pigs (1940c); *S. anatum* from a horse (1940b). With Hayston (1941) he isolated *S. dublin* from calves. Hayston (1942) described further strains of *S. cholerae-suis* var. *kunzendorf* from pigs. Hindmarsh (1940) also recovered *S. typhi-murium* from sheep. Many workers have isolated *S. pullorum* from poultry.

Apart from infections with *S. typhi*, we found only two accounts of Salmonellas isolated from man. In one case (Frank, 1937) the organism incriminated was considered to be *S. enteritidis* although complete serological typing was not carried out. In the other case the organism proved to be a new type and was called *S. adelaide* (Atkinson, 1943).

By antigenic analysis the Salmonellas have been divided into at least 114 types. The importance of this typing lies in its epidemiological application. From this point of view Edwards and Bruner (1943) and Bornstein (1943) have studied the occurrence of the types in the United States. No such studies have been made in this country, and we wish to determine the type and incidence of strains occurring in Australia.[2]

## SEROLOGICAL METHODS.

After establishing the smoothness of the culture by testing with trypaflavin, we investigated the O antigens by slide agglutination with a series of O antisera. We examined the H antigens of a highly motile culture by tube agglutination tests with a group H antiserum (prepared against *S. cholerae-suis* var. *kunzendorf*); if no H agglutination occurred the organism was then tested for the other factors of Phase 2 and the factors of Phase 1. With diphasic organisms, a suspension was prepared in each phase and tested with the appropriate antisera. When necessary we tested the organisms against absorbed sera specific for a given antigen, and occasionally did agglutinin absorptions.

Most of our cultures belonged to Group B of the Kauffmann-White classification, many being *S. typhi-murium*. The O antigen IV characterizes this group, all of which also possess XII; some members also possess I or V or I and V. Edwards and Bruner (1939) showed that these variable antigens were useful in grouping cultures of *S. typhi-murium* according to their origin. We tested our cultures of Group B for I by slide agglutination with an O antiserum prepared against *S. paratyphi A* I, II, XII and absorbed with *S. typhi* IX, XII. We did not consider the

[1] Working with a grant from the University of Adelaide.

[2] We now possess the wide range of antisera needed for complete typing of a Salmonella; We are willing to type any cultures sent to us for identification. In fact we can perform the functions of a "Salmonella Centre" as set up in various parts of the United States, but the final reference for unusual problems is the International Salmonella Centre under the direction of Dr. Kauffmann at Copenhagen. We shall be pleased to render this service to any workers who have neither the time nor the inclination to type their Salmonellas; in this way we hope to collect representative specimens of the types occurring in Australia.

presence of II in the serum likely to confuse the result as this factor occurs only in *S. paratyphi A*, and is difficult to eliminate. To test for V we used an O serum prepared against *S. typhi-murium* IV, V, XII and absorbed with *S. typhi-murium* var. *copenhagen* IV, XII.

## RESULTS.

*The Types of Salmonella and their Antigenic Analysis.*

All cultures, except one, belonged to Group B of the Kauffmann-White classification. They included *S. typhi-murium*, *S. reading*, *S. derby*, *S. stanley* and *S. paratyphi B*. The exception was a new Salmonella with an O antigen. It was named *S. adelaide* with suggested antigenic formula XXXV: fg . . . (Atkinson, 1943).

Among our cultures of *S. typhi-murium* all possible O antigenic types occurred (these are IV; I, IV; I, IV, V; IV, V). The specific H antigen i showed the usual minor overlap with r as described by Kauffmann (1939).

The cultures of *S. reading* were identified by the antigens of Phase 2. The O antigen IV and the H antigens eh were present and the organism was diphasic possessing the group antigen 1. This analysis showed that the culture was either *S. reading* IV: eh: 1, 5 or *S. kaapstad* IV: eh: 1, 7. A suspension in Phase 2 agglutinated with antiserum specific for 5 but not with one specific for 7. The cultures were therefore *S. reading*.

Our cultures of *S. derby* contained both I and IV and were identified by agglutination with a specific f serum and by agglutinin absorption tests.

The culture of *S. stanley* was identified by its specific H antigen d.

The cultures of *S. paratyphi B* were identical in antigenic structure. All contained O antigens IV and V but not I, and were diphasic in their H antigens, Phase 1 containing b and Phase 2 the group antigen 1. This last antigen was responsible for the final identification as the other organism with similar O and Phase 1 antigens, *S. abony* (I), IV, V: b: enx, differs in the antigens of Phase 2.

*The Origin and Frequency of Occurrence of the Salmonella Types.*

We examined a total of 46 different strains[3] of which 35 were *S. typhi-murium*, two *S. reading*, three *S. derby*, one *S. stanley*, four *S. paratyphi B* and one *S. adelaide*.

Of the 35 strains of *S. typhi-murium* 24 were isolated from the faeces of infants and children with various diseases. The majority showed symptoms of gastro-enteritis, and two died. Four strains were obtained from faeces of adults with gastro-enteritis, one of whom died, and from whose liver the organism was then isolated. Two strains came from healthy carriers. The remaining cultures were of heterogeneous origin, one from a cat with enteritis then epidemic in cats, one from "spanish cream" which caused a small outbreak of food-poisoning, one from the liver of a turkey which died after eating "spanish cream". Two cultures, previously unidentified, which had been maintained among the stocks since 1930, had been isolated from cases of food-poisoning.

Edwards and Bruner (1939) pointed out the epidemiological significance of the antigenic sub-groups of *S. typhi-murium*. They found that in animal infections all cultures from one outbreak contained the same O antigens. Christensen (1937) working with human strains, found all cultures from one oubreak identical in containing or lacking antigen I. Most of our cultures were isolated singly, but a few groups were related. One such group comprised four cultures isolated in the "spanish cream" outbreak—one from the "spanish cream", two from infected adults and one from an infected turkey. The culture from one adult contained I and IV, the others contained only IV. A strain from one child who died and another strain from her mother, who gave no history of gastro-enteritis, also differed antigenically. The former was I, IV, V and the latter IV. Two strains isolated from two brothers, one with gastro-enteritis, the other apparently healthy, also differed antigenically, one containing IV and the other I, IV. If the antigenic type is of epidemiological significance, the carrier in the family in the last two cases was not responsible for the child's infection. In the "spanish cream" outbreak, either more than one type of *S. typhi-murium* was present in the food without our detecting the fact, or one of the adults acquired the infection elsewhere.

The majority of our cultures were IV variants, which Edwards and Bruner (1939) found almost exclusively in pigeons and which never contained I. Many of our IV variants contained I and thus resembled Christensen's (1937) strains of human origin, in 50 p.c. of which he found antigen I. These workers found a correlation between antigenic structure and fermentation tests in bitter medium with maltose, xylose and rhamnose. In these conditions all their IV variants were poor fermenters. Our IV variants differed in this respect also from theirs, the majority giving a positive test with all three substrates (see Table 1). We conclude that our IV variants are a different type.

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[3] We wish to thank Mr. Draper of the Elder Laboratory, Adelaide Children's Hospital, for supplying many of these cultures.

TABLE 1.

*Source and reactions of strains of* S. typhi-murium.

| Source of strain. | Number of strains tested. | Bitter test. Maltose. | Xylose. | Rhamnose. | Somatic antigens. |
|---|---|---|---|---|---|
| Children with enteritis or other complaints | 3 | + | + | + | I, IV, V |
| | 1 | + | − | + | I, IV, V |
| | 1 | + | + | + | IV, V |
| | 1 | − | − | + | IV, V |
| | 11 | + | + | + | I, IV |
| | 4 | + | + | + | IV |
| Carriers | 1 | + | + | − | IV |
| | 1 | + | + | + | IV |
| Adults with enteritis | 1 | + | − | + | I, IV, V |
| | 2 | − | − | + | IV, V |
| | 1 | + | + | + | I, IV |
| | 1 | + | + | + | IV |
| | 1 | + | − | + | IV |
| Cat with enteritis | 1 | + | − | + | IV |
| "Spanish cream" | 1 | + | + | + | IV |
| Turkey liver | 1 | + | + | + | IV |

Both our strains of *S. reading* and two of *S derby* came from children with gastro-enteritis: the third strain of *S. derby* came from an adult with ulcerative colitis.

The culture of *S. stanley* was isolated in 1928 from a case of food-poisoning and had been maintained unidentified among the stock-cultures.

The four strains of *S. paratyphi B* came from one outbreak which involved about 20 persons, one of whom died after a short severe illness diagnosed as acute enteritis. The other patients suffered only a mild attack, all recovering in three to seven days. All except the patient who died had been inoculated with T.A.B. vaccine, which may have been responsible for their mild reaction. All the strains of *S. paratyphi B* were identical biochemically and antigenically.

The one strain of *S. adelaide* was isolated from an adult who died after a short illness diagnosed as severe enteritis. The organism was obtained from the faeces during life and from the liver and spleen after death.

## DISCUSSION.

A survey of the Salmonellas occurring in Australia and its environs is long overdue. The typing of Salmonellas, however, is a specialized technique requiring the use of a wide range of cultures and antisera. Until these were available the survey was impossible. We are now able and willing to type any Salmonellas sent to us. The importance of determining the types and their distribution lies in the epidemiological application of the results.

As a beginning we present Table 2, which incorporates all the accurate data which we could obtain to date on the Salmonellas in Australia. We have not included *S. pullorum*, frequently recovered from poultry, nor *S. typhi*, as typhoid fever is endemic in Australia.

The preponderance and wide distribution of *S. typhi-murium* agree with the findings elsewhere. Edwards and Bruner (1943) found approximately 1,000 strains of *S. typhi-murium* among 3,090 Salmonellas investigated in the United States. They found it in man and in 24 varieties of domestic and fur-bearing animals. With the exception of *S. paratyphi B* it occurred in man more frequently than any other type. The IV variant, however, was seldom found in man and the majority of their strains came from pigeons. In this respect our results differ from theirs as we found IV variants predominated among our human strains.

TABLE 2.

*Salmonellas recorded in Australia.**

| Salmonella. | Source. | Types of illness. | Number affected. | Reference. |
|---|---|---|---|---|
| | *New South Wales.* | | | |
| *S. typhi-murium* | sheep | dysentery | about 400 | Hindmarsh (1940). |
| | sheep | not named | 17 | Stewart (1940a). |
| | pigs | enteritis and pneumonia | about 80 | Stewart (1940b). |
| | horses | acute septicaemia | 3 | Stewart (1940b). |
| | ducks | not named | 2,000 | Stewart (1939). |
| | *South Australia.* | | | |
| | turkey | enteritis | 1 | Present paper. |
| | cat | enteritis | numerous | Present paper. |
| | "spanish cream" | — | 1 | Present paper. |
| | children | enteritis | 24 | Present paper. |
| | adults | enteritis | 6 | Present paper. |
| | human carriers | none | 2 | Present paper. |
| | *New South Wales.* | | | |
| *S. cholerae-suis* var. *kunzendorf* | pigs | unnamed | 10 | Stewart (1940c). |
| | pigs | pneumonia | 37 | Hayston (1942). |
| | *New South Wales.* | | | |
| *S. bovis morbificans* | sheep | unnamed | 200 | Stewart (1940a). |
| | pigs | pneumonia | 1 | Stewart (1940b). |
| | *New South Wales.* | | | |
| *S. dublin* | calves | dysentery and fever | 3 | Stewart and Hayston (1941). |
| | *New South Wales.* | | | |
| *S. anatum* | horses | acute septicaemia | 1 | Stewart (1940b). |
| | *Victoria.* | | | |
| *S. enteritidis* | adult | enteritis | 1 | Frank (1937). |
| | *South Australia.* | | | |
| *S. stanley* | adult | enteritis | 1 | Present paper. |
| | *South Australia.* | | | |
| *S. derby* | children | enteritis | 2 | Present paper. |
| | adult | ulcerative colitis | 1 | Present paper. |
| | *South Australia.* | | | |
| *S. reading* | children | enteritis | 20 | Present paper. |
| | *Northern Territory.* | | | |
| *S. paratyphi B* | adults | enteritis | 2 | Present paper. |
| | *South Australia.* | | | |
| *S. adelaide* | adult | enteritis | 1 | Atkinson (1937). |

* On account of the inadequacy of the methods of identification of the Salmonellas concerned, earlier references appearing in the Australian literature have not been included in this Table. Mainly the organisms were reported as *Bacillus paratyphosum* A and B and *Bacillus aertrycke* A and B.

These workers and others have stressed the epidemiological importance of the presence or absence of antigen I in all strains from a single outbreak. We had only 8 strains definitely related to each other, which relationship was not indicated by antigen I. The other Salmonellas in Table 2 are well-known types except for *S. adelaide*, which has only been found once.

It is interesting that among our small number of cultures from human cases we obtained three strains of *S. derby* and two of *S. reading*, whereas Edwards and Bruner (1943) found among 492 cultures isolated from man only four *S. derby* and no *S. reading*, and Bornstein (1943) at the New York Salmonella Centre obtained out of 500 cultures from man 11 *S. derby* and no *S. reading*. Edwards and Bruner (1943) frequently isolated *S. derby* from fowls and swine, but only one culture of *S. reading*, obtained from a pig, occurred among the 3,090 examined. Two other Salmonellas not found in this American study were *S. bovis-morbificans* and *S. stanley*. In Australia the former occurs in sheep and pigs and the latter was found in one case of food-poisoning. Bornstein (1943) also did not find *S. stanley* among his 500 cultures from man, but records one case of *S. bovis-morbificans* causing septicaemia.

More than half our cultures came from children who are apparently more susceptible to infection than adults.

## SUMMARY.

Of 46 local strains of Salmonella typed in this laboratory 35 were *S. typhi-murium*, two *S. reading*, three *S. derby*, one *S. stanley*, four *S. paratyphi B* and one *S. adelaide*.

Among the strains of *S. typhi-murium*, which were mainly of human origin, IV variants predominated.

A Table is given to show the incidence of Salmonellas so far described in Australia.

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# ATTEMPTS TO TRANSMIT LEUCAEMIA OF MAN AND OF MICE TO THE CHICK EMBRYO AND TO THE YOUNG CHICK BY THE AMNIOTIC AND INTRAVENOUS ROUTES

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The discovery of Ellermann and Bang (1908) that fowl leucosis can be transmitted with cell-free filtrates from diseased birds to healthy ones was later followed by successful transmissions to near relatives of the fowl by Stubbs (1933) to fowl-pheasant hybrids, by Engelbreth-Holm and Rothe-Meyer (1932) to guinea fowls, and by Jarmai (1935) to the turkey. Even interspecies transmissions were later reported by Pollard and Hall (1942) with material from spleen, liver and kidneys from affected chick embryos after inoculation into embryos of ducks, guinea-fowl, pheasants and quails. The way to these experiments was prepared by Engelbreth-Holm and Rothe-Meyer (1932), Jarmai (1935) and Pierce (1942a) who found no difficulty in transmitting avian leucosis to the chick embryo with cellular material.

As leucaemia in mammals is regarded by many as being essentially the same disease process as fowl leucosis, the differences being mainly due to the different species, it was of great interest to use the chick embryo to study a possible relationship between the two. Pierce (1942b) cultivated human leucaemic leucocytes on the chorio-allantoic membrane of the chicken embryo. However, no leucosis developed 4-5 months after the chickens hatched. Pierce used blood and bone marrow from 16 leucaemias, of which 11 were acute, 2 subacute, 2 chronic and one was a chloroma. The inoculation of the chick embryo was made between the 9th and 13th day of incubation, and the period of observation was between 2 and 14 days after the inoculation.

During 1941-42, the same period when Pierce conducted her experiments, similar experiments were independently conducted here. After numerous preliminary studies of inoculants of the chorio-allantoic membrane in 1939, experiments were undertaken to use the amniotic sac or the intravenous route with the human leucaemic material.

Later experiments with leucaemic material from mice were conducted, and material was re-inoculated in young mice of an AK strain after three chick-embryo passages.

## ATTEMPTS TO TRANSMIT HUMAN LEUCAEMIA TO THE CHICK EMBRYO.

Human leucaemic blood, obtained by sterile venepuncture with citrated saline as anticoagulant, was injected into the amniotic sac of 11-day old chick embryos, using Burnet's method. After four days of further embryonic life the embryo was aseptically removed from the egg; one lung, spleen, and bone marrow from one femur and tibia were ground up with sterile sand and amniotic fluid. The cellular emulsion thus obtained was used for further passages into the amniotic sac of 11-day old chick embryos. The other lung, the bone marrow of the other tibia and femur, and a blood film were examined histologically for evidence of leucaemic lesions. Blood agar plates were inoculated with the human blood originally used for the injections and with the emulsions used for the chick embryo passages; the material was discarded whenever it was

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proved to be contaminated by bacteria. Only experiments with sterile material are reported here.

The passages were made although evidence of transmission was lacking in numerous preliminary trials in which only one inoculation was done and followed up with post-mortem examinations of the chick embryo from the 12th to the 20th day of incubation. The passages were continued on the hypothesis that it might take several passages for the human agent to get accustomed to the chick embryo.

Chick embryos and chicks raised at the South Australian Government poultry station served as controls. The strain of White Leghorn has been free from spontaneous leucaemia for the last 12 years. More than 10,000 chickens are reared every season, coming from the same birds which laid the eggs used in these experiments.

*Experiment A.* Five attempts were made with blood, and one with bone marrow taken from human cases of chronic myeloid leucaemia (see Table 1, A). At least two passages, and, on two occasions, four passages, were made. Usually 9 to 12 chick embryos, sometimes more, were used for the first inoculation; for later passages 8 to 10 embryos were usually inoculated. They showed no leucaemic reaction or evidence of the presence of a leucaemic agent. In order to observe the possible development of later leucaemic reactions further experiments were conducted.

TABLE 1.

*Attempts at transmission of human leucaemia to the chick embryo and young chick.*

| Patient. | Age. | Sex. | Type of leucaemia. | White blood count. | Material used for inoculation. | Differential count of leucaemic cells in p.c. Polymorphs. | Myelocytes. | Myeloblasts. | No. of inoculations. | No. of passages. | Live chicks. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A. | | | | | | | | | | | |
| N. | 36 | M | Chronic myeloid | 254,000 | Blood | 26 | 45 | 29 | 11 | 4 | — |
| Ha. | 19 | F | „ „ | 454,000 | „ | 43 | 53 | 4 | 14 | 4 | — |
| Ha. | 19 | F | „ „ | 200,000 | „ | 49 | 48 | 3 | 12 | 2 | — |
| H. | 37 | M | „ „ | 300,000 | „ | 27 | 32 | 41 | 11 | 2 | — |
| McR. | 36 | F | „ „ | 108,000 | Sternal marrow | 54 | 39 | 7 | 13 | 2 | — |
| McR. | 36 | F | „ „ | 180,000 | Blood | 46 | 42 | 12 | 12 | 2 | — |
| B. | | | | | | | | | | | |
| N. | 36 | M | Chronic myeloid | 154,000 | Blood | 60 | 29 | 11 | 64 | – | 6 |
| P. | 36 | F | „ „ | 105,000 | „ | 30 | 59 | 11 | 24 | – | 3 |
| E. | 14 | F | Acute myeloid | 174,000 | „ | 1 | — | 99 | 20 | – | 3 |
| C. | | | | | | | | | | | |
| E. | 14 | F | Acute myeloid | 75,000 | Blood | 1 | 4 | 95 | 21 | – | 21 |
| C. | 62 | M | Chronic myeloid | 250,000 | „ | 47 | 44 | 9 | 21 | – | 21 |
| LeG. | 34 | M | „ „ | 365,000 | „ | 42 | 50 | 8 | 21 | – | 21 |
| D. | | | | | | | | | | | |
| C. | 32 | F | Acute myeloid | 50,000 | Blood | 2 | — | 98 | 13 | 5 | — |
| A.a | 15 | F | „ „ | 100,000 | „ | 15 | — | 85 | 17 | 2 | — |
| A.b | 15 | F | „ „ | 165,000 | Bone marrow | 1 | — | 99 | 17 | 2 | — |
| T. | 18 | F | „ „ | 70,000 | Blood | 1 | — | 99 | 18 | 5 | — |
| P.a | 50 | F | „ „ | 104,000 | „ | 7 | — | 93 | 16 | 2 | — |
| P.b | 50 | F | „ „ | 164,000 | Bone marrow | 1 | — | 99 | 18 | 2 | — |
| E.a | 14 | F | „ „ | 31,000 | Blood | 15 | — | 85 | — | 2 | — |
| E.b | 14 | F | „ „ | 100,000 | „ | 3 | — | 97 | — | 2 | — |
| E.c | 14 | F | „ „ | 175,000 | Bone marrow | 1 | — | 99 | — | 3 | — |

*Experiment B.* Chick embryos inoculated into the amniotic sac with leucaemic material on the 11th day of embryonic life were permitted to hatch. Other embryos received leucaemic material into the umbilical vein: these experiments gave a very high death rate, the majority of chick embryos dying in the shell on the 20th day of incubation (Table 1, B).

Of the 12 chickens which hatched, the three inoculated with material from E. were quite normal on autopsy at the age of 8 months. Of the three chickens raised after inoculation with material from patient P., two developed "big bones" within six months; and of the six chickens

raised after the inoculation with material from patient N. three developed "big bones" within six months. The 10,000 chickens during the same season at the Government Poultry Station served as controls, none of which had developed "big bones" for several years in the past, or in this year.

The onset of the skeletal changes was insidious, became noticeable in the first month and had fully developed in six months. The sexual distribution was four males and one pullet. The pullet and two affected cockerels were kept for breeding, but no evidence of a hereditary factor in the 40 resulting chicks was found, this generation being apparently perfectly normal after one year of observation.

*Experiment C.* Hatched chickens one day old and one week old were injected with human leucaemic material in an attempt to obtain similar lesions (Table 1, C). Of these 63 chickens, none developed any big bones or leucaemic reactions during the next 12 months. It appears therefore that the "big bones" can be produced only by injection into the chick embryo at a very sensitive stage of its development.

**Fig. 1.** X-ray photograph of the tubular bones showing the osteopathia hyperostotica sclerotieans multiplex infantilis.

**Fig. 2.** Photograph of the involved bones.

The "big bone disease" obtained in the experiments consisted in a general enlargement in width of the bones, due to a hyperostotic sclerosis; the normal bone marrow had disappeared and was replaced by a very dense lamellar bone with only small medullary cavities. This new bone formation not only filled the cavities but also extended along the bone peripherally like a large spindle, and gave the legs a plump appearance. The gait of these birds was rather stiff and ungraceful. The bone marrow, liver, spleen and nerves gave no indication of leucaemic infiltrations or reactions. This disease in fowls was seen in previous years in a few rare cases in the stock of the Parafield poultry station and was described by G. C. McLennan as bone disease of fowls simulating Paget's disease (unpublished report). It involved only the long bones, generally affected only young birds, developed within the first 6 months of life and according to McLennan "was sometimes associated with an anaemia characterized by a low red cell count, high white blood cell count and low haemoglobin. The blood phosphatase was found to be 0·25 as compared with a normal of 0·6 in one case".

Intense sclerosis of the marrow of tubular bones has been described by Ellerman in birds with myeloid leucaemia, but was also produced experimentally by McGowan by intraperitoneal injections of finely powdered glass. I would, therefore, consider the "big bones" in the above described experiments as not a specific lesion and evidence of fowl leucaemia, but as a reaction of the bone to the injection of the leucaemic material at a sensitive stage of development. However, further investigations might throw more light on this problem.

## ATTEMPTS AT TRANSMISSION WITH MATERIAL FROM ACUTE MYELOID LEUCAEMIA.

The transmission experiments with material from acute myeloid leucaemia (Table 1, D) sometimes gave lesions in the lungs of the chick embryos in the first passage. A mild irritation of the lung was found similar to that in the early stages of infection of the chick embryo with influenza virus. This, however, was not a constant finding, and could not be produced regularly in a significantly high percentage of the experiments. Of ten chicks which were permitted to hatch after inoculation with material from case P., two developed in the first week of life a leucaemoid blood picture. This incidence was singular, and its importance is doubtful, as it is well known that fowls often show leucaemic reaction in controls as well as in experimental birds, and that one has to be careful with the interpretation of results.

## ATTEMPTS TO TRANSMIT LEUCAEMIA OF MICE INTO CHICKENS AND BACK TO MICE.

Blood and emulsion of a spleen of a mouse 12 months old (strain AK) of a known spontaneous lymphoid leucaemia were inoculated into the chick embryo on the amniotic sac. The chick embryos were 11 days old and were killed at 14 and 20 days. No evidence of reaction in blood, lung, spleen or bone marrow of the chick embryo was found in three lots of six chickens.

This experiment was then repeated. The chick embryos were killed on the 15th day and a second and third chick embryo passage made. Again on the 15th day of incubation of the chick embryo, four days after inoculation of the leucaemic mice splenic material, the chick embryos were aseptically dissected. Spleen, bone marrow and lung were ground up with sterile sand and saline. The resulting emulsion was then injected intraperitoneally and subcutaneously into 18 mice of the known strain AK (from J. Furth). For this re-inoculation of the AK strain 5 litters were split, half of each being used as controls and half as experimental animals (half intraperitoneally, half subcutaneously). (Mice born 1.12.40, injected 3.1.41, killed 15.3.41.) Leucaemia failed to develop within three months after the inoculation of these mice. The controls were in no way different from the experimental animals in the composition of the W.B.C., differential W.B.C. count, the size of spleen or liver.

In a similar way, material from a human acute leucaemia was injected after a chick-embryo passage (with lesions in the lung) into 20 mice of strain AK, using the other half (20) of the litters as controls. (Mice born 4 and 17.3.1941, inoculated 16.5.41, killed 23.9.41.) Again no difference was noted in the blood and organs of the controls and the experimental mice.

## SUMMARY.

Leucosis did not develop in the chick embryo, young chick or adult fowl following the inoculation of the chick embryo with human leucaemic material using the amniotic or intravenous route.

An osteopathia hyperostotica scleroticans multiplex infantilis developed in 5 fowls out of 12 which hatched after they were inoculated intravenously with human material from chronic leucaemias on the 11th day of their embryonic life.

Human material from acute leucaemias inoculated into the 11-day-old chick embryo in a small proportion of cases produced in the lungs early lesions similar to those in influenza virus infection. This lesion is not regarded as specific.

Leucosis did not develop in the young chick or adult fowl after intravenous injection of the young chick on the first and seventh day of life with human material from chronic and acute cases of leucaemia.

Mouse leucaemia (AK strain, J. J. Furth) produced no leucaemia after inoculation into the same AK strain of mice after 3 chick-embryo passages when the amniotic route was used.

Material from an acute case of human leucaemia produced lesions in the lung of the chick embryo but did not produce a leucaemic reaction in AK strain of mice after a chick-embryo passage.

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